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Super-Organismal Effects Of A Widespread Insect Endosymbiotic Bacteria

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Abstract

Ant colonies are a hub of diverse interactions that are affected by a multitude of factors, such as colony members, external environment, and possibly the symbiotic bacteria that live within individual colony members. While symbiotic microbes are well-known to manipulate the physiology, ecology and evolutionary biology of their solitary hosts, we have limited understanding of their effects on the biology of social insects such as ants. Wolbachia, a maternally-inherited endosymbiont, is the most widespread insect endosymbiont. It manipulates host reproduction and confers fitness benefits to the host to favor its own vertical transmission. While Wolbachia is known to manipulate the reproductive biology of their solitary hosts, we know practically nothing about its effects in social species. In my thesis, I have compared Wolbachia-infected and uninfected colonies of the pharaoh ant, Monomorium pharaonis. I show that Wolbachia-infected colonies have higher colony growth and reproductive investment that arise due to individual-level differences in the queens. Wolbachia infection doesn't seem to exact a detectable cost. Given these effects, Wolbachia can rapidly spread through colonies. Thus, Wolbachia infection rate has the potential increase even in natural populations, although this may be limited by the trade-offs that can become evident in certain conditions. Results from my thesis bridge a critical gap in our understanding of the effects of a widespread bacterial endosymbiont on the life history of a superorganism.

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ABSTRACT

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Ant colonies are a hub of diverse interactions that are affected by a multitude of factors, such as colony members, external environment, and possibly the symbiotic bacteria that live within individual colony members. While symbiotic microbes are well-known to manipulate the physiology, ecology and evolutionary biology of their solitary hosts, we have limited understanding of their effects on the biology of social insects such as ants. *Wolbachia*, a maternally-inherited endosymbiont, is the most widespread insect endosymbiont. It manipulates host reproduction and confers fitness benefits to the host to favor its own vertical transmission. While *Wolbachia* is known to manipulate the reproductive biology of their solitary hosts, we know practically nothing about its effects in social species. In my thesis, I have compared *Wolbachia*-infected and uninfected colonies of the pharaoh ant, *Monomorium pharaonis*. I show that *Wolbachia*-infected colonies have higher colony growth and reproductive investment that arise due to individual-level differences in the queens. *Wolbachia* infection doesn't seem to exact a detectable cost. Given these effects, *Wolbachia* can rapidly spread through colonies. Thus, *Wolbachia* infection rate has the potential increase even in natural populations, although this may be limited by the trade-offs that can become evident in

certain conditions. Results from my thesis bridge a critical gap in our understanding of the effects of a widespread bacterial endosymbiont on the life history of a superorganism.

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CHAPTER 1: Introduction

Symbiotic bacteria in solitary species

All multicellular organisms engage in symbiotic interactions with free-living bacteria that reside within the host, so much that we are said to be living in a bacterial world (McFall-Ngai et al. 2013). The host and bacterial pathways extensively crosstalk with each other and regulate each other's traits and fitness (Dillon and Dillon 2004; Engel and Moran 2013). Symbiotic bacteria can affect a variety of individual-level traits of its host, such as development (Sommer and Bäckhed 2013), immunity (Hooper, Littman, and Macpherson 2012), neurological function (Sampson and Mazmanian 2015), and nutrition and metabolism (Douglas 2009; Nicholson et al. 2012). Symbiotic bacteria can also regulate the social behavior and interactions of their solitary hosts (Archie and Theis 2011; Archie and Tung 2015), e.g., mating preference (Sharon et al. 2010), kin-recognition (Lizé, McKay, and Lewis 2014; Lewis et al. 2014), pheromonal communication (Ezenwa and Williams 2014), and social attraction (Venu et al. 2014). Such effects can also contribute to host speciation (Shropshire and Bordenstein 2016).

Endosymbiotic bacteria are non-free living symbiotic bacteria that exclusively reside within the cells of their host. They have played a very critical role in the major evolutionary transition from a prokaryotic life to a eukaryotic life (Archibald 2015; Martin, Garg, and Zimorski 2015). Lynn Margulis (Sagan) proposed in her landmark paper in 1967 that "..*mitochondria, the (9 + 2) basal bodies of the flagella, and the photosynthetic plastids can all be considered to have derived from free-living cells, and the eukaryotic cell is the result of the evolution of ancient symbioses*" (Sagan 1967). Studies from

multiple solitary species, such as aphids, fruit flies, solitary wasps, and mosquitoes, continue to point towards the profound effects of endosymbionts on the physiology, ecology, and evolutionary biology of their hosts.

Wolbachia as a reproductive manipulator

Wolbachia, an alphaproteobacterium, is the most prevalent endosymbiont that infects an estimated 60% of terrestrial insects (Sazama et al. 2017), although incidence within insect populations can either be very low (<10%) or very high (>90%) (Hilgenboecker et al. 2008). *Wolbachia* strains are divided into 16 monophyletic clusters called supergroups which are labelled from A-Q (Lo, Casiraghi, and Salati 2002; S. Bordenstein and Rosengaus 2005; Pascar and Chandler 2018; Vera I. D. Ros et al. 2009) and each consists of multiple strains that were identified based on the sequence divergence of five ubiquitous genes (Baldo et al. 2006). Each supergroup may induce different phenotypic effects on its host and/or maybe associated with different hosts. For example, Supergroup A and B are facultative endosymbionts in insects, i.e., insect populations may or may not be infected, and have a wide variety of phenotypic effects that range from being parasitic to mutualistic to the host (Werren, Baldo, and Clark 2008). Whereas, Supergroup C and D are obligate endosymbionts of the filarial nematodes that are necessary for host survival (Taylor, Bandi, and Hoerauf 2005).

Wolbachia is maternally-inherited within a species and it can manipulate the reproductive biology of its host to favor its vertical transmission. It does so by inducing either unidirectional or bidirectional reproductive incompatibility between infected and uninfected mates or by killing infected males since males can’t transfer *Wolbachia* to the

next generation or by feminizing infected females or by resulting in parthenogenesis of infected females (Jan Engelstädter and Hurst 2009; Landmann 2019; Zug and Hammerstein 2014). In addition to these, *Wolbachia* infection may induce beneficial phenotypes such as increasing female fecundity (Fast et al. 2011; Dedeine et al. 2001), providing protection against RNA viruses (Hedges et al. 2008; Luís Teixeira, Ferreira, and Ashburner 2008), and vitamin B and iron provisioning (Nikoh et al. 2014; Hosokawa et al. 2010; Brownlie et al. 2009). It should be noted that *Wolbachia*-induced phenotypes are conditional on multiple factors, such as *Wolbachia* strain, host species, and environmental conditions (A. J. Fry, Palmer, and Rand 2004; Hague et al. 2020; Zélé, Santos, et al. 2020; L. Mouton et al. 2006; Laurence Mouton et al. 2007). For example, *Wolbachia* strain *w*Ri increases the basal activity levels, improves the responsiveness to food cues, and improves the olfactory response of *Drosophila simulans* by increasing the expression of the olfactory receptor gene *or83b*, (Peng and Wang 2009; Peng et al. 2008). In contrast, *Wolbachia* strains *w*Mel and *w*MelPop do not have such effects in *Drosophila melanogaster* and *w*MelPop had very little effect on the responsiveness of *Drosophila simulans* to food cues (Peng et al. 2008). Given these phenotypic manipulations of the hosts, *Wolbachia* can rapidly spread through the host population (Kriesner and Hoffmann 2018; Kriesner et al. 2013; M. Turelli and Hoffmann 1991; Michael Turelli et al. 2018; Jansen, Turelli, and Godfray 2008; Bakovic et al. 2018; Schuler et al. 2016).

Wolbachia also has the potential to manipulate social interactions in solitary species. For example, it regulates cuticular hydrocarbons in *Drosophila melanogaster*

that can affect communication between developing male and female pupae (Pontier and Schweisguth 2015). However, effects of *Wolbachia* on the phenotypes of highly social organisms, such as ants, are not well characterized.

Endosymbionts in eusocial insects

Eusocial insects, such as ants, honey bees, and termites, epitomize social living and pose a unique challenge for reproductive manipulators such as *Wolbachia*. Eusociality is characterized by reproductive division of labor, overlapping generations within colonies, and cooperative brood care (Hölldobler and Wilson 1990). The colonies are a hub of diverse interactions amongst nestmates that drive colony-level outputs. The colonies also offer a great potential for endosymbiotic bacteria, such as *Wolbachia,* to regulate not only individual-level traits but also colony-level traits, such as caste allocation, colony growth, and worker foraging, to facilitate its own vertical transmission. Furthermore, endosymbionts that manipulate host reproduction are proposed to be one of the drivers for the evolution of haplodiploidy, an important feature of the life cycle of eusocial insects (Normark 2004; J. Engelstädter and Hurst 2006).

One of the best characterized endosymbiont in social insects is *Blochmannia*, which is commonly found in the Camponotini tribe (Wernegreen et al. 2009). *Blochmannia* is an obligate endosymbiont that resides in specialized bacteriocytes (Sauer, Dudaczek, and Hölldobler 2002), provides nutrition to its host (Feldhaar et al. 2007) and is critical for embryonic development (Rafiqi, Rajakumar, and Abouheif 2020). *Blochmannia* has been able to hijack its host's embryonic development for its vertical transmission by evolving as a key driver that alters the expression of *Hox* genes to

regulate germline development in the embryos (Rafiqi, Rajakumar, and Abouheif 2020). However, *Blochmannia* is not the only ant endosymbiont and certainly not a widespread endosymbiont in ants. While endosymbionts have been implicated to affect group-level traits, their roles are underexplored (Koch and Schmid-Hempel 2011; Engel and Moran 2013; Russell, Dubilier, and Rudgers 2014)

Wolbachia-ant association

Wolbachia infects an estimated 34% of ant species, although its effects on the individual- and colony-levels phenotypes of the host largely remain unknown (Russell 2012). Ants are commonly infected with multiple strains of *Wolbachia*, either from Supergroup A or B or both (Russell 2012; Andersen et al. 2012; Tseng et al. 2019; Bouwma et al. 2006; Russell et al. 2012). *Wolbachia* localises in both the germline and somatic cells of individual queens and workers, even though *Wolbachia* is transmitted only by the queens and the workers are an evolutionary dead end (Frost et al. 2014; Andersen et al. 2012; Ramalho et al. 2018). At the population-level, *Wolbachia* infection rates are affected by the dispersal strategy of the colonies. Infection is prevalent in populations with limited queen dispersal and dependent colony foundation (queens and workers bud away from an existing colony and disperse a short distance to establish a new nest) compared to populations with independent colony foundation (queen disperse longer distance to establish a new colony by herself) (Treanor and Hughes 2019; Russell 2012; Tsoi 2013). Thus, *Wolbachia* has the potential to affect the population structure of ants. *Wolbachia* prevalence in populations is also affected by invasion. Infection is lost in the invading populations of the Argentine ants (*Linepithema humile;* (Reuter, Pedersen,

and Keller 2005; Tsutsui et al. 2003)), fire ants (*Solenopsis invicta;* (Shoemaker et al. 2000; Bouwma et al. 2006)), and little fire ant (*Wasmannia auropunctata*; (Rey et al. 2013)) compared to their native populations, suggesting that *Wolbachia* may either trade-off with invasiveness or is lost in response to the new habitat. In some cases, *Wolbachia* can be detrimental to the ant host. For example, colonies of *Cardiocondyla obscurior* that are infected with different strains of *Wolbachia* have reduced reproductive output due to mating incompatibility with each other (Ün et al. 2020). Infected colonies of *Formica truncorum* produce less number of new queens and males which is expected to reduce colony growth (Wenseleers, Sundström, and Billen 2002). However, in the ghost ant, *Tapinoma melanocephalum*, *Wolbachia* is known as a nutritional symbiont as it provides vitamin B (Cheng et al. 2019). Furthermore, as we showed previously, *Wolbachia*-infected *Monomorium pharaonis* colonies have a queen-biased sex ratio that may facilitate *Wolbachia*'s vertical transmission and may also increase colony growth (L. Pontieri et al. 2017). However, there is limited knowledge about the individual- and colony-level effects of *Wolbachia*, especially across the colony life cycle. This paucity in evidence largely exists because of the difficulty to manipulate and track ant colonies for generations and limited standing variation in colony-level *Wolbachia* infection in the samples colonies.

Monomorium pharaonis **as a study system**

The pharaoh ant, *Monomorium pharaonis*, overcomes these shortcomings to emerge as a tractable system. *M. pharaonis* is an extremely successful global invasive pest that is present on all continents except Antarctica (Wetterer 2010). The colonies can

be maintained and bred in the laboratory for generations, the colony life cycle can be reduced to approximately 6 weeks, and most importantly for my thesis, colonies show natural differences in the *Wolbachia* infections status.

As part of a long term research program, eight different *Monomorium pharaonis* colonies were collected from eight different geographical locations around the world (A. M. Schmidt 2010). Since then, these colonies have been systematically interbred for nine generations to result in over hundred genetically diverse colonies (J. T. Walsh, Garnier, and Linksvayer 2020; A. M. Schmidt 2010). Two of these eight colonies were naturally infected with *Wolbachia* (A. M. Schmidt 2010). Given the maternal inheritance of *Wolbachia*, multiple descendent colonies were also naturally infected. Furthermore, given the extensive crossing scheme, we expect *Wolbachia* infection to be relatively decoupled from the genotypes of the colonies (Singh and Linksvayer 2020).

Monomorium pharaonis colony life cycle begins with the eclosion of new queens and males, followed by intra-colony matings and an investment in colony productivity. Queens are the only egg-laying caste in the colonies as workers are obligately sterile (Michael R. Warner, Lipponen, and Linksvayer 2018; Hölldobler and Wilson 1990). Once the queens die or are reproductively senile, the workers can rear new queens and males from the existing batch of eggs in a colony, which will start a new colony life cycle (Fowler, Alves, and Bueno 1993).

Ant colony growth and reproduction pose a unique challenge for *Wolbachia* since they are regulated by different colony members (Cassill et al. 2005; A. M. Schmidt et al. 2011; M. R. Warner, Kovaka, and Linksvayer 2016; Beros et al. 2019) and can affect the

Wolbachia-induced phenotypes. Specifically, in *M. pharaonis* colonies, queens are the only egg laying reproductive females, whereas the obligately sterile workers forage for food, share the food with nestmates, and nurse younger developmental stages (Børgesen 1989; Edwards 1991; Fowler, Alves, and Bueno 1993). Even the late-instar larvae in colonies contribute and regulate colony growth. They are responsible for processing solid proteins which are regurgitated by the late-instar larvae and shared with colony members to boost queen fecundity (M. R. Warner, Kovaka, and Linksvayer 2016; Edwards 1987; Børgesen and Jensen 1995; Børgesen 1989; Edwards 1991; A. M. Schmidt et al. 2011; Michael R. Warner, Lipponen, and Linksvayer 2018). Colony members can also regulate the colony demography and caste allocation. The queens lay eggs of different reproductive fate, such as queens versus workers versus males, depending on environment and food availability, workers selectively cull queen- and male-destined eggs, and late-instar larvae provide digested proteins which can boost production of new queens and males (M. R. Warner, Kovaka, and Linksvayer 2016; Michael R. Warner, Lipponen, and Linksvayer 2018; Edwards 1991, 1987; A. M. Schmidt et al. 2011; Børgesen 1989; Oliveira et al. 2020; Børgesen and Jensen 1995).

Thesis outline

In my thesis, I provide a detailed characterization of the *Wolbachia*-induced benefits and costs, both at an individual- and colony-level. In **Chapter 1**, I review the evolutionary importance of endosymbiosis and discuss the current paucity of research to understand the endosymbiosis in ants. Following this I highlight the limited nature of direct evidence for *Wolbachia*-induced phenotypes in ants. In **Chapter 2,** I first establish

colony-level fitness differences by comparing naturally infected and uninfected *Monomorium pharaonis* colonies at discrete time points and across the colony's life cycle. I show that *Wolbachia*-infected colonies have higher growth rates and reproductive investment and increased reproductive senescence which can lead to shorter colony life cycle length. These effects suggested that *Wolbachia* may be enhancing colony-level fitness which may also incur a cost. In **Chapter 3,** I elucidate the individual-level differences in the queens that contribute to colony-level growth differences and the underlying cost of simultaneously maintaining higher colony growth and *Wolbachia*. I show that infected queens have increased egg-laying rates early in their life span that may directly contribute to increased colony growth. This increased egg laying did not exact a detectable energetic cost and did not trade-off with the lifespan of the queens. Interestingly, infected workers outlived uninfected workers. Thus, *Wolbachia* may increase colony growth rates by increasing egg laying rates of the queens and the lifespan of the adult workers. These effects, coupled with the absence of detectable cost, may facilitate the spread of infection through colonies and populations that have both infected and uninfected members. In **Chapter 4**, I test the evolutionary consequences of the phenotypic effects of *Wolbachia* in *M. pharaonis* colonies. I compare the within-colony infection and life cycle dynamics of colonies that consist of both infected and uninfected members over a period of two years. The *Wolbachia* infection rate increased in such mixed colonies within two years that span approximated four generations of *M. pharaonis* colonies. Furthermore, these colonies also produced more queens as the infection increased. Although we did not see signs of early reproductive senescence since the colony life cycle was similar between infected, uninfected and

mixed colonies. In my last chapter, **Chapter 5,** I provide closing arguments by discussing the scope and limitations of my results and the future research that will be helpful to better understand *Wolbachia*-ant association.

Overall my thesis is the first to provide direct evidence of *Wolbachia*-induced phenotypes in ants. It also characterizes the basic biology of *Monomorium pharaonis* and establishes *Monomorium pharaonis* as a viable study system to explore the social effects of symbiotic relationships.

CHAPTER 2: *Wolbachia*-infected ant colonies have increased reproductive investment and an accelerated life cycle

Abstract

Wolbachia is a widespread group of maternally-transmitted endosymbiotic bacteria that often manipulates the reproductive strategy and life history of its hosts to favor its own transmission. *Wolbachia* mediated phenotypic effects are well characterized in solitary hosts, but effects in social hosts are unclear. The invasive pharaoh ant, *Monomorium pharaonis*, shows natural variation in *Wolbachia* infection between colonies and can be readily bred under laboratory conditions. We previously showed that *Wolbachia*-infected pharaoh ant colonies had more queen-biased sex ratios than uninfected colonies, which is expected to favor the spread of maternally-transmitted *Wolbachia*. Here, we further characterize the effects of *Wolbachia* on the short- and longer-term reproductive and life history traits of pharaoh ant colonies. First, we characterized the reproductive differences between naturally infected and uninfected colonies at three discrete time points and found that infected colonies had higher reproductive investment (i.e. infected colonies produced more new queens), in particular when existing colony queens were three months old. Next, we compared the long-term growth and reproduction dynamics of infected and uninfected colonies across their whole life cycle. Infected colonies had increased colony-level growth and early colony reproduction, resulting in a shorter colony life cycle, when compared to uninfected colonies.

Introduction

Wolbachia, a maternally-inherited group of endosymbiotic bacteria, is considered to be the most prevalent endosymbiotic bacteria in arthropods (Weinert et al. 2015; Sazama et al. 2017; Sazama, Ouellette, and Wesner 2019). Infection has a range of effects on host reproduction, including reproductive incompatibility between infected males and uninfected females, reproductive incompatibility between mates infected with different strains of *Wolbachia*, female-biased sex ratios in offspring of infected females, killing of infected males (Jan Engelstädter and Hurst 2009; Zug and Hammerstein 2014; Landmann 2019), and increased fecundity of infected females (Fast et al. 2011; Weeks et al. 2007; A. J. Fry, Palmer, and Rand 2004). These manipulations of host reproduction by *Wolbachia* are expected to facilitate its own spread in the host populations, even when the manipulation is costly to the host (Kriesner et al. 2013; Schuler et al. 2016; Jiggins 2017; Bakovic et al. 2018; Kriesner and Hoffmann 2018; Michael Turelli et al. 2018; Jansen, Turelli, and Godfray 2008).

Effects of *Wolbachia* on host reproduction vary across host species, ranging from beneficial to detrimental (Jan Engelstädter and Hurst 2009; Zug and Hammerstein 2014; Landmann 2019). For example, *Wolbachia* influences the pheromone profile of infected fruit flies which in turn affects mating success (Pontier and Schweisguth 2015) and gamete compatibility (Schneider et al. 2019). In *Drosophila paulistorum, Wolbachia* is required for the production of male sexual pheromones for successful mating (Schneider et al. 2019), However, in the case of *Drosophila simulans*, *Wolbachia* regulates the pheromonal communication between male and female pupae during metamorphosis,

which affects gametic compatibility between infected and uninfected adult mates (Pontier and Schweisguth 2015). These examples also illustrate that *Wolbachia* can affect traits that influence social interactions in solitary species, suggesting that *Wolbachia* could also affect various individual- and group-level traits of highly social hosts such as ants.

Wolbachia is estimated to infect 34% of ant species (Russell 2012), localizing in the germline and various somatic tissues of the worker and queen ants (Andersen et al. 2012; Frost et al. 2014; Sapountzis et al. 2015; Zhukova et al. 2017; Ramalho et al. 2018). Across ant species, *Wolbachia* infection is correlated with colony reproductive strategy, with higher incidence in colonies with dependent colony foundation i.e., when new colonies are established by a group consisting of single or multiple mated queens and some workers, compared to independent colony foundation, where single queens establish new colonies (Wenseleers et al. 1998; Russell 2012; Treanor and Hughes 2019). Interestingly, invasive populations of the Argentine ant (*Linepithema humile*) and the fire ant (*Solenopsis invicta*) show a marked population-wide reduction of infection compared to their native populations (Tsutsui et al. 2003; Reuter, Pedersen, and Keller 2005; Shoemaker et al. 2000; Bouwma et al. 2006). Furthermore, in the ghost ant (*Tapinoma melanocephalum*), *Wolbachia* plays a role in Vitamin B provisioning (Cheng et al. 2019). However, the specific individual- and colony-level effects of *Wolbachia* infection in ants, especially on the reproduction and growth of ant colonies, remain largely unknown.

The invasive pharaoh ant, *Monomorium pharaonis*, is one of the most successful and well-studied invasive ants (Wetterer 2010). Most importantly for the current study, pharaoh ant colonies show natural variation in *Wolbachia* infection status (A. M. Schmidt 2010; L. Pontieri et al. 2017). We previously showed that *Wolbachia*-infected pharaoh ant colonies produced fewer males and had a queen-baised sex ratio (relative number of new queens versus males produced by a colony) when artificially selected for higher caste ratio (relative number of new queens versus workers) across three generations (L. Pontieri et al. 2017). Since queens are the only reproductive caste in pharaoh ant colonies, such a queen-biased investment is expected to increase the transmission and prevalence of maternally-inherited *Wolbachia*. This also suggests that *Wolbachia* may manipulate colony reproduction and life cycle to increase its own transmission from one generation to the next.

In the current study, we provide a detailed characterization of differences in the reproduction, life cycle, and life history of pharaoh ant colonies that show natural variation in *Wolbachia* infection in the absence of artificial selection. The pharaoh ant colony life cycle begins with intra-colony matings between newly produced males and queens, followed by the production of only sterile workers, and ends with the spontaneous production of new queens and males when the existing queens senesce after approximately four months (Fowler, Alves, and Bueno 1993). Henceforth, we define this spontaneous production of new queens and males as colony reproduction and we use the counts of queen and male pupae as a proxy to measure colony reproduction. We predict *Wolbachia*-infected colonies to have an increased investment

in queens, as workers are obligately sterile and *Wolbachia* is maternally transmitted. Such a queen-biased investment is expected to affect the colony-level productivity and life cycle dynamics. We designed two separate assays to compare the (a) colony-level reproductive investment at discrete time points (i.e. queen ages), and (b) long-term colony life cycle dynamics in the absence of disturbance (Fig. 2.1).

Materials and methods

Source of infected and uninfected colonies

We sought to construct replicate experimental colonies that had known *Wolbachia* infection status (i.e. were either infected or uninfected), but were genetically homogeneous. Briefly, as part of a long-term research program, we have systematically intercrossed eight pharaoh ant lineages, originally collected from locations around the world, for nine generations, in order to create a population of genetically heterogeneous lab colonies, henceforth called heterogeneous stock colonies (Fig. S2.1a; (J. Walsh et al. 2019; A. M. Schmidt 2010; L. Pontieri et al. 2017)). Two out of the eight initial lineages were infected with *Wolbachia* (A. M. Schmidt 2010), and based on the known pedigree of colonies in our lab population, we also putatively know the *Wolbachia* infection status of these colonies (because *Wolbachia* is maternally inherited; Fig. S2.1a). We empirically verified the expected infection status of heterogeneous stock colonies in the lab by screening five individual workers per colony using a previously described PCR-based method (Baldo et al. 2006). Nine generations of systematic intercrossing is expected to result in a population of colonies where genetic background is relatively uncoupled from *Wolbachia* infection status (Fig. S2.1b; permutation test, P =

0.46). That is, infected colonies, which have maternal parentage from one or both of the two infected lineages are expected to possess a similar genetic makeup as uninfected colonies, which have paternal parentage from the two infected lineages but only have maternal parentage from the six uninfected lineages (Fig S2.1b; Supplementary file S2.1; (Anna M. Schmidt, d'Ettorre, and Pedersen 2010; A. M. Schmidt 2010; L. Pontieri et al. 2017)).

In order to create two sources of known infection status that were relatively genetically homogeneous, we combined 15 of these heterogeneous stock colonies that were infected by *Wolbachia*, and separately combined 14 colonies that were uninfected by *Wolbachia* (note that M. pharaonis colonies readily merge after a period of transient aggression that lasts less than one day (Luigi Pontieri 2014)). We subsequently used these two sources to create replicate experimental colonies of known infection status (see Assay 1 and Assay 2 below).

In order to synchronize the age of queens in these source colonies, we induced the production of new queens and males, i.e., colony reproduction, by removing all the existing queens (Edwards 1987, 1991; A. M. Schmidt et al. 2011; M. R. Warner, Kovaka, and Linksvayer 2016; Michael R. Warner, Lipponen, and Linksvayer 2018). Workers in such queenless colonies are expected to rear new adult queens and males from the existing pool of eggs. We periodically examined these source colonies and removed any new spontaneously produced reproductive larvae/pupae over the course of our experiments to ensure that all queens in these source colonies were the same age. All colonies were maintained in environmental growth chambers at 27 ± 1°C, 50% RH and

12:12 LD cycle and were fed ad libitum synthetic agar diet (sugar:protein = 3:1; (Dussutour and Simpson 2008)) and dried mealworms (*Tenebrio molitor*) twice a week.

Quantifying differences in colony growth and reproduction dynamics

We compared productivity and life cycle differences between *Wolbachia*-infected and uninfected pharaoh ant colonies using two assays. In Assay 1 we compared the differences in reproductive investment at three discrete time points. In Assay 2 we compared the differences in colony productivity and colony life cycle dynamics of the pharaoh ant.

Assay 1: Reproductive investment of colonies at discrete time points

In Assay 1, we measured the total number of new queens and males produced by ten replicate infected and seven replicate uninfected colonies across three discrete time points (i.e. when queens were 1- 3- and 6-months old) that span the reproductive lifespan of the queens. We created similarly sized replicate experimental colonies of known infection status with no queens and with approximately 500 workers and 500 brood (eggs, larvae, and pupae). These queenless experimental colonies were kept for ten days during which all eggs transitioned to older developmental stages since pharaoh ant workers are obligatorily sterile and can't lay eggs (Fig 2.1; (Hölldobler and Wilson 1990)). Once these queenless experimental colonies were eggless, we added 20 age-matched queens from source colonies to these experimental colonies for only 48 hours (Fig. 2.1). We added known-aged infected queens from infected source colonies only to infected experimental colonies and known-aged uninfected queens from

uninfected source colonies to uninfected experimental colonies. After 48 hours, we transferred these queens back to their respective source colonies and we censused the number of eggs laid by these queens (Fig. 2.1). These experimental queenless colonies now contained eggs from age-matched queens and were kept until eggs developed into new worker, male, and queen pupae (approximately 35 days). We censused the number of new worker, male, and queen pupae produced 29 and 35 days after adding age-matched queens to the experimental colonies. We summed these two censuses to calculate the total number of worker, male, and queen pupae produced by each replicate colony. We used these total counts to compute the relative investment in new queens versus workers, i.e., colony caste ratio (L. Pontieri et al. 2017). We also computed the relative investment in new queens versus males, i.e., colony sex ratio (L. Pontieri et al. 2017). Note that we used a blind design, where we were blind to the infection status of colonies for data collection.

Assay 2: Colony growth, reproduction, and life cycle dynamics

In Assay 2, we tracked 14 infected and 12 uninfected experimental colonies for seven months in order to compare the (a) colony productivity, both workers and reproductives, and (b) colony life cycle dynamics of naturally infected and uninfected colonies across the colony life cycle.

We created similarly sized queenless and eggless experimental colonies, with approximately 500 workers and 500 brood (eggs, larvae, and pupae) in the same manner as described for Assay 1. Once eggless, we added 20 one-month-old infected queens from the infected source colonies to each infected experimental colony and 20

one-month-old uninfected queens from uninfected source colonies to each uninfected replicate experimental colonies (Fig. 2.1). We censused the colonies after 48h of adding queens to quantify initial colony composition, and we did not manipulate the colonies any further. The queens aged naturally in these colonies and we surveyed the colony composition across the whole colony life cycle on a monthly basis. Specifically, for the first four months we counted each developmental stage, from eggs to pupae, and reproductive adults (Fig. 2.1). After four months, the colonies were sizable and it was difficult to get accurate counts of younger developmental stages. Hence, after four months we restricted the counts to new male and queen pupae and adults, and worker pupae (Fig. 2.1). At each time point, we calculated net colony productivity as the total number of pupae (workers, queens and males) present at the time of census (Fig. 2.1). We did not compute caste and sex ratio for these colonies in Assay 2 as they grew to very different sizes and variation in colony size is known to affect colony caste ratio (A. M. Schmidt et al. 2011). We used a blind design for data acquisition, where we were blind to the colony infection status at the time of census.

We also assessed differences in worker body mass between infected and uninfected colonies over a period of time in Assay 2. We collected 15 early stage worker pupae from each replicate colony after two, three, four, and six months from the beginning of the assay. We identified early stage worker pupae as those with white bodies and pigmented eyes (Linksvayer 2006). We dried these pupae at 55^{o}C for 20 hours before storing them at -20^{o}C till the time of weighing them on Sartorius

microbalance (MSU3.6P-000-DM) in milligrams up to three decimal points. We used a blind design for data collection.

Statistical analysis

We used R version 3.5.2 (R Core Team 2019), with lme4 (Bates et al. 2015), pscl (Zeileis, Kleiber, and Jackman 2008), MASS (Venables and Ripley 2002), and car packages (Fox and Weisberg 2019) for data analysis, and ggplot2 (Wickham 2016) for plotting graphs. We built generalized linear mixed effect models (GLMM; (Bolker et al. 2009)) to assess the overall effects of predictor variables (*Wolbachia* infection and queen age) on response variables (fitness traits such as total number of queens, sex ratio, and caste ratio), with source colonies as a random factor. We performed a post-hoc TukeyHSD test on GLMM for pairwise comparison of response variables across queen-age or time. To assess the effect of *Wolbachia*-by-queen age (Assay 1) or *Wolbachia*-by-time (Assay 2) interaction on colony-level phenotypic traits, we used generalized linear models (GLMs; (Bolker et al. 2009)) with *Wolbachia* infection, queen age/time, and *Wolbachia*-by-queen age/time interaction as fixed factors. To compare infected and uninfected colonies at specific time points, we used GLMs. For count data, we constructed GLMMs with Poisson and GLMs with negative binomial or quasi-Poisson error distributions. For caste and sex ratio, we constructed GLMMs assuming binomial and GLMs assuming quasi-binomial error distributions. Since larger colonies tend to invest relatively more in new workers versus new queens in terms of caste ratio when compared to smaller colonies (A. M. Schmidt et al. 2011), we included log-transformed colony productivity (i.e. total number of new workers, queens, and males produced, as a

measure of colony size) as a fixed factor when assessing caste and sex ratio differences in Assay 1. In Assay 2, experimental colonies produced new males only between 4 and 7 months after starting the assay. We compared the differences in production of male pupae between infected and uninfected colonies during this period. For assessing differences in dry weight of worker pupae collected in Assay 2, we used linear mixed effects models (LMM; (Galecki and Burzykowski 2013)) with mean dry mass per colony as the response variable, *Wolbachia*-by-time interaction as a fixed factor, log-transformed colony productivity as a fixed factor, and colony ID as a random factor. For age-specific effects of *Wolbachia* infection, we constructed LMM with mean dry mass per colony at a specific time point as the response variable, *Wolbachia* as a fixed factor, log-transformed colony productivity as a fixed factor, and colony ID as a random factor. We computed the statistical significance of each component of the LMM model via Anova from the car package (Fox and Weisberg 2019). Datasets for Assay 1, Assay 2, and genetic relatedness are included in supplementary excel files (S1-S3). R scripts and output from statistical models are available on Dryad. See the 'Data Availability' section for more details.

Results

Assay 1: Wolbachia-infected colonies had higher queen production and reproductive investment

Overall in Assay 1, *Wolbachia*-infected colonies produced more queen pupae (GLMM; LRT = 8.62, $p = 0.003$; Fig. 2.2a) and had queen-biased caste ratios (GLMM; LRT = 5.95, $p = 0.014$; Fig. 2.2c) and sex ratios (GLMM; LRT = 4.65, $p = 0.041$; Fig.

2.2d). In particular, *Wolbachia*-infected experimental colonies with 3-month-old queens produced more new queens (GLM: F = 5.63, p = 0.031; Fig. 2.2a) but a similar number of males (GLMM; LRT = 0.03, p = 0.84; Fig. 2.2b), resulting in a queen-biased caste ratio (GLM: F = 9.01, p = 0.009; Fig. 2.2c) in these colonies.

In addition to *Wolbachia* infection, queen age also affected colony-level traits. The total number of eggs present in the experimental colonies after 48h increased with queen age (GLMM; F = 1421.15, p < 0.001; Fig. S2.2a). The total number of new queens produced from these eggs was also dependent on maternal age (GLMM: LRT = 419, p < 0.001), specifically, colonies with 3-month-old queens produced the most new queens (GLM: z < 18, p < 0.001; Fig. S2.2b). Furthermore, all colonies with older queens produced more males (GLMM: LRT = 224.48, p < 0.001; Fig. S2.2c) and workers (GLMM: LRT = 1767.97, p < 0.001; Fig. S2.2d). Specifically, experimental colonies with 6-month-old queens had male-biased sex ratios (GLMM: LRT = 130.35, p < 0.001; Fig. S2.2e) and worker-biased caste ratios (GLMM: LRT = 579.27, p < 0.001; Fig. S2.2f).

Assay 2: Wolbachia-infected colonies have increased colony-level growth, early colony reproduction, and faster colony life cycle.

Across the colony lifespan, *Wolbachia*-infected colonies overall produced more new workers (GLMM: LRT = 6.7, p = 0.009; Fig. 2.3a), had a non-significant trend towards more new queens (GLMM: LRT = 3.46, p = 0.062; Fig. 2.3b), and produced a similar number of males (GLMM: LRT = 1.76, p = 0.18; Fig. 2.3c) relative to uninfected colonies. Interestingly, *Wolbachia*-infected colonies spontaneously produced new

queens and males earlier than uninfected colonies (Fig. 2.3b, 2.3c). At specific time points, infected colonies had more total number of queens after four months (GLM: F = 13.25, p = 0.001) and five months (GLM: F = 12.44, p = 0.001; Fig. 2.3b) of starting the assay, relative to uninfected colonies at the same points. Similarly, infected colonies produced more males after four months (GLM: LRT = 7.81, p = 0.02) and five months (GLM: LRT = 9.03, p = 0.01; Fig. 2.3c) of starting the assay, relative to uninfected colonies at the same time points. This is in contrast with uninfected colonies that seem to spontaneously produce new queens and males approximately after six months (Fig. 2.3b and 2.3c). Furthermore, *Wolbachia*-infected colonies had increased worker productivity after two months (GLM: F = 8.76, p = 0.007), six months (GLM: F = 6.4, p = 0.019), and seven months (GLM: F = 6.38, p = 0.019) of starting the assay relative to uninfected colonies at the same time point. Interestingly, infected and uninfected colonies produced a similar number of eggs (GLMM: LRT = 0.4, p = 0.51; Fig. S2.3a), although infected colonies had more late-instar larvae relative to uninfected colonies after two months of starting the assay (GLM: F = 4.85, p = 0.039; Fig. S2.3b). The dry mass of *Wolbachia*-infected worker pupae was also dependent on time (LMM: X^2 = 17.76, $p < 0.001$; Fig. S2.3c) and infected worker pupae were heavier after two months of starting the assay (LMM: F =8.72, p = 0.007; Fig. S2.3c). While colony productivity was not a major predictor of worker pupae dry weight differences across all time points (LMM: X^2 = 1.21, p = 0.27), it however, was a major predictor of differences in dry weight after six months of starting the assay (LMM: F =5.91, p = 0.02).

Discussion

In the current study we provide a detailed characterization of differences in productivity, reproductive investment, and life cycle dynamics of pharaoh ant colonies that had similar genotypes but differed in *Wolbachia* infection status. *Wolbachia*-infected pharaoh ant colonies have a reproductive (Fig. 2.2, 2.3b, 2.3c) and growth (Fig. 2.3a) advantage that is dependent on the age of the queens (Assay 1) and time or stage of the colony life cycle (Assay 2). Furthermore, infected colonies spontaneously produced new reproductives (i.e. new queens and males) earlier than uninfected colonies (Fig. 2.3b and 2.3c). Usually, the presence of reproductively fecund queens in pharaoh ant colonies suppress the production of new queens and males (Michael R. Warner, Lipponen, and Linksvayer 2018; Edwards 1987, 1991; Fowler, Alves, and Bueno 1993). Hence the spontaneous production of new reproductives suggests that *Wolbachia*-infected queens may experience early reproductive senescence compared to uninfected queens. While we did not directly quantify queen mortality, a steady increase in worker and queen numbers over a period (Fig. 2.3a, b) suggest that new queens were being added even when some of the old queens were still alive in the colonies (Fig. 2.3b). These results point to accelerated colony life cycle dynamics, and possibly an alternate life history strategy for *Wolbachia*-infected queens.

Increased growth and accelerated life cycle of *Wolbachia*-infected pharaoh ant colonies is expected to increase colony size and the frequency of colony reproduction (i.e. decrease the generation time) relative to uninfected colonies, which is expected to be favorable in expanding populations. Invasive species such as pharaoh ants likely find

themselves in conditions where such rapid population expansion is favored, e.g., following invasion into a new habitat. New pharaoh ant colonies are established when some of the existing queens and workers “bud” off from the sufficiently large parent colony and occupy new nest sites (Fowler, Alves, and Bueno 1993; Buczkowski and Bennett 2009). *Wolbachia*-infected colonies may possibly have a higher frequency of such colony-founding events, which may increase their invasiveness. Moreover, rapid expansion of *Wolbachia*-infected pharaoh ant colonies may also result in increased prevalence of *Wolbachia*. Infection can sweep through a host population if there is a growth advantage to the host or manipulation of host reproduction by *Wolbachia* (Jansen, Turelli, and Godfray 2008; Kriesner and Hoffmann 2018). Future experiments mapping the incidence of *Wolbachia* in the invasive population of pharaoh ants across the globe will be insightful.

The probability of infection sweeping through pharaoh ant populations and a concomitant increase in the invasiveness of *Wolbachia*-infected populations, can be expected to depend on multiple factors such as environmental conditions, frequency and type of inter-colony interactions, and also intra-colony interactions. For example, *Wolbachia* density in hosts is sensitive to ambient temperatures and it decreases with either increase or decrease in temperatures (Hurst et al. 2000; S. R. Bordenstein and Bordenstein 2011). Thus, it is possible that fluctuating environmental temperatures may affect *Wolbachia* density in ant hosts and hence limit the subsequent phenotypic effects and potential fitness advantages of infected pharaoh ant colonies. Furthermore, competition between colonies for nest space, food, and other resources may also limit

the propagation of infected pharaoh ant colonies. Ant colony growth and reproduction is socially regulated, i.e., different members of the colony regulate colony growth and reproduction (Aron, Keller, and Passera 2001; Clark et al. 2006; Schmickl and Karsai 2018; M. R. Warner, Kovaka, and Linksvayer 2016; Penick and Liebig 2012), including regulation of caste development in colonies by workers (Michael R. Warner, Lipponen, and Linksvayer 2018), regulation of queen development by workers (Clark et al. 2006; Penick and Liebig 2012) , and the importance of late-instar larvae for the production of new queens and males (M. R. Warner, Kovaka, and Linksvayer 2016). Hence, interactions within and between colonies, possibly in response to environment or amongst nest mates of differing infection status, may also affect the spread of *Wolbachia*. In the wild, rapidly expanding invasive and *Wolbachia*-infected pharaoh ant colonies will likely come in contact with both infected and uninfected colonies. Pharaoh ant colonies show transient inter-colony aggression, and colonies in the laboratory readily merge despite being highly genetically differentiated (Luigi Pontieri 2014). However, it is uncertain how frequently and readily colonies merge in the wild (Anna M. Schmidt, d'Ettorre, and Pedersen 2010). Future studies simulating such scenarios with both *Wolbachia*-infected and uninfected individuals within the same colony will further elucidate the dynamics of *Wolbachia* sweeping through colonies and populations.

In a previous study where we artificially selected for increased or decreased caste ratio (i.e. increased or decreased investment in new queens relative to workers) in replicate populations across three generations, we found that *Wolbachia*-infected colonies had queen-biased sex ratios, specifically due to decreased male production (L.

Pontieri et al. 2017). In the current study, we similarly observed that infected colonies invested relatively more in new queens (i.e. we observed increased queen production, queen-biased caste ratios, and queen-biased sex ratios), but infected colonies did not produce fewer males. Thus, both studies point to female-biased sex allocation differences associated with *Wolbachia*-infection that are expected to favor the spread of *Wolbachia*, and the specific differences between our current and previous studies could have resulted due to small differences in genetic sources used or in environmental conditions (e.g., differences in nutrition, temperature, or humidity) between the two studies.

The differences between *Wolbachia*-infected and uninfected colonies that we observed, while similar to the phenotypic effects of *Wolbachia* infection in solitary species, are expected to arise partly from mechanisms fairly unique to social organisms. For example, infected pharaoh ant colonies produced more pupae (Fig. 2.3a) but a similar number of eggs (Fig. S2.3a) compared to uninfected colonies. This suggests that infected colonies have a higher egg-to-pupa survival. This could be attributed to either individual-level differences in the quality of the eggs laid by the queens or the collective differences in foraging and nursing behaviors of infected workers, or both. These differences could also possibly be due to the beneficial nutritional provisioning by *Wolbachia*, as *Wolbachia* has been shown to be a nutritional mutualist in other insects (Brownlie et al. 2009; Nikoh et al. 2014; Hosokawa et al. 2010), including the ghost ant, *Tapinoma melanocephalum* (Cheng et al. 2019). Future studies investigating possible

nutritional symbiosis between *Wolbachia* and pharaoh ant queens and its implication on the viability of brood and adults will be insightful.

In summary, we show novel productivity and life history differences between pharaoh ant colonies showing natural differences in *Wolbachia* infection. *Wolbachia*-infected queens and colonies had an accelerated life cycle that may be favored as an alternate life history strategy. Such effects may be beneficial for the rapid expansion of invasive pharaoh ant colonies and for the increased spread of *Wolbachia* in populations. Our results also underscore the importance of queen age when comparing colony fitness and life cycle dynamics. Overall, our research shows that the pharaoh ant, *Monomorium pharaonis*, is a tractable, highly social system for studying the effects of *Wolbachia* across generations. Future studies are necessary to tease apart the specific mechanisms by which *Wolbachia* manipulates individual- and colony-level traits. These include directly studying the lifespan of *Wolbachia* infected and uninfected queens as well as comparing physiological correlates of aging and reproductive senescence (Negroni, Foitzik, and Feldmeyer 2019; Keller and Jemielity 2006; Corona et al. 2007)

Data availability

The census data for Assay 1 and Assay 2, the dry mass of worker pupae, and the relatedness values among heterogeneous lab stock colonies are included as supplementary files accompanying this manuscript. The R scripts used for analysis in the article and the output from statistical models can be accessed at Dryad (https://doi.org/10.5061/dryad.tht76hdw5).

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Figures

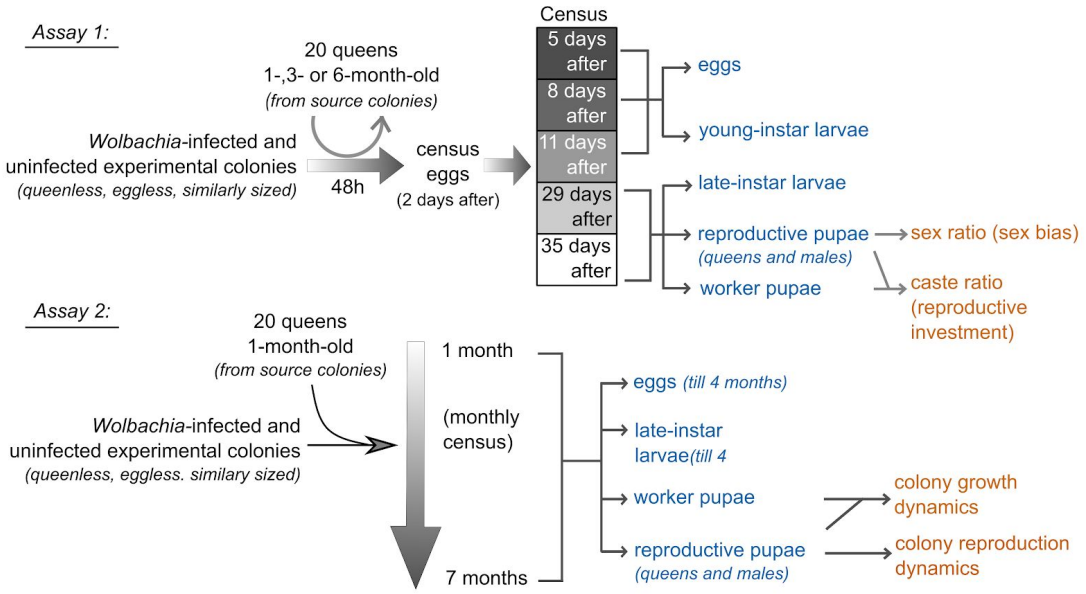

Figure 2.1. Schematic description of Assay 1 and Assay 2 for measuring the effects of *Wolbachia* infection status on productivity, reproduction, and life cycle of pharaoh ant colonies. We used Assay 1 (top) to assess colony-level reproductive investment at discrete queen ages and Assay 2 (bottom) to follow colony life cycle dynamics over time. We censused different ant development stages (in blue) at various times (arrows on the left of the development stages) to compute colony-level traits (orange) from various combinations of these census values (arrows on the right of the development stages).

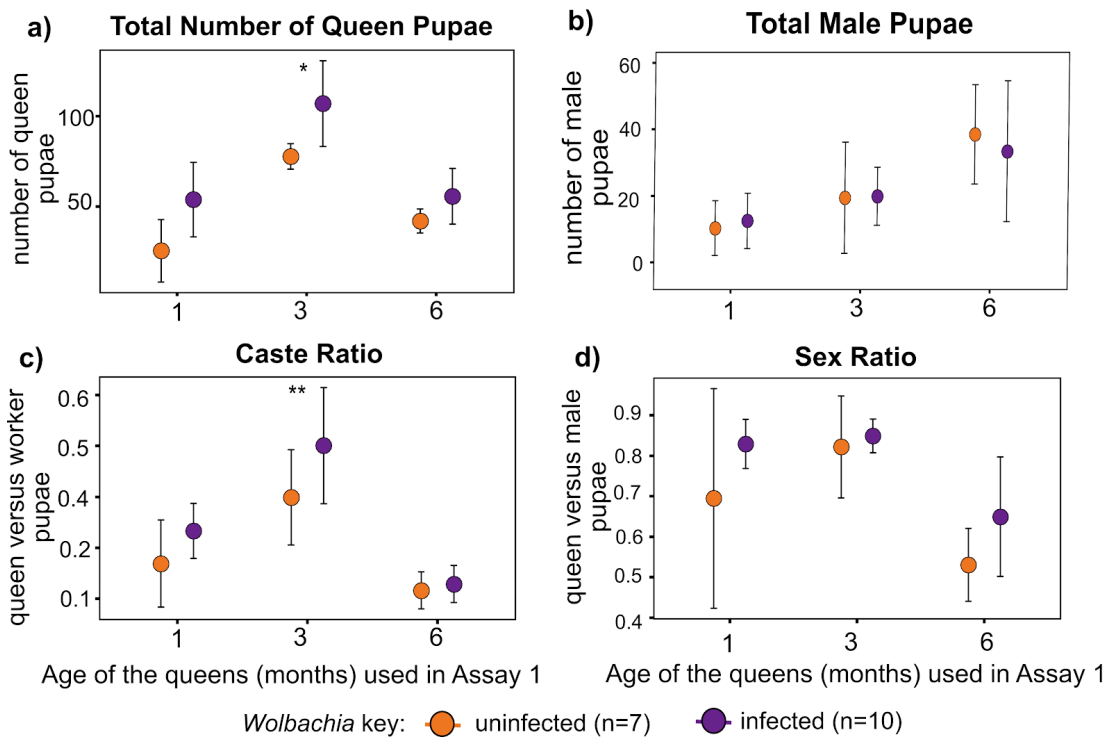

Figure 2.2: *Wolbachia* increases reproductive investment of pharaoh ant colonies, depending on queen age. (a) Infected colonies produced more queen pupae when queens used for the assay were 3-month-old. (b) No differences in the total number of male pupae produced by infected and uninfected colonies. (c) Infected colonies have increased queen-biased caste ratio when queens used for the assay were 3-month-old. (d) *Wolbachia*-infected colonies show a non-significant trend towards queen-biased sex ratio. Filled circles represent the mean trait value and error bars represent the 95% confidence interval of the mean. *Wolbachia*-related differences are represented as $p < 0.05$* and <0.01**, and were estimated by age-specific GLMs. The number (n) of replicate colonies in the assay are at the bottom of the figure panel.

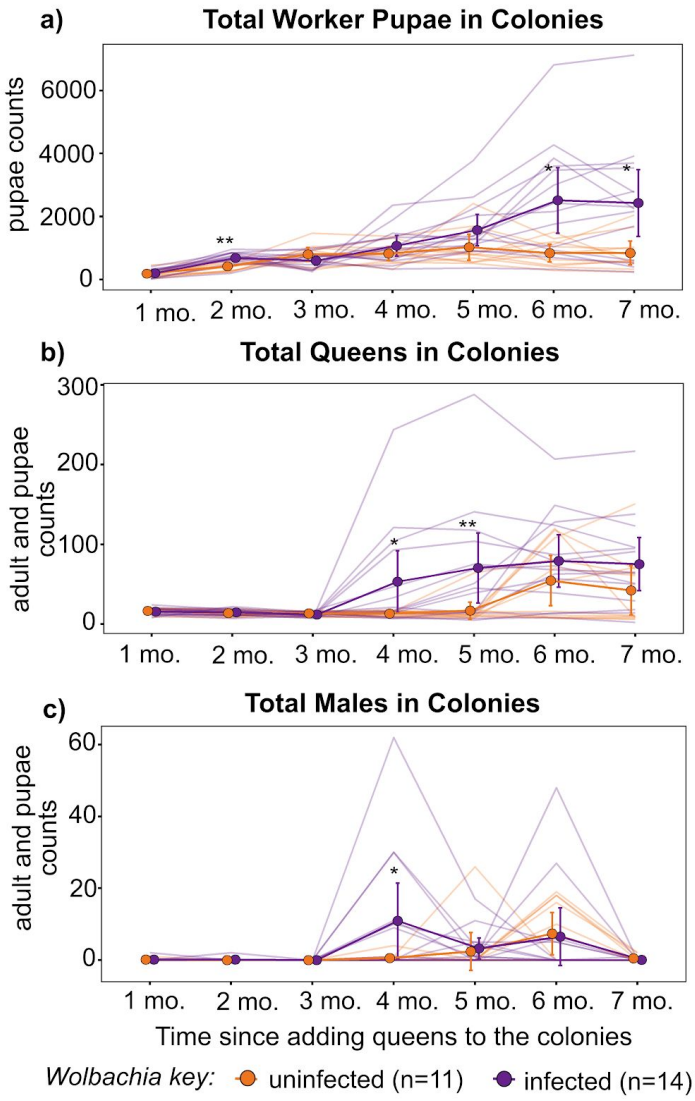

Figure 2.3: Infected colonies had increased growth and early onset of reproduction. (a) Infected colonies produced more pupae two months after starting the assay. (b) Infected colonies had an early spontaneous production of new queens. (c) Infected colonies had an early spontaneous production of new males. Filled circles represent the mean trait value and error bar represents the 95% confidence interval of

the mean. Light-colored lines represent individual colony-level values. *Wolbachia*-driven differences are represented as $p < 0.05$* and <0.01**, and were estimated by age-specific GLM. The number (n) of replicate colonies in the assay are at the bottom of the figure panel.

Supplementary Figures

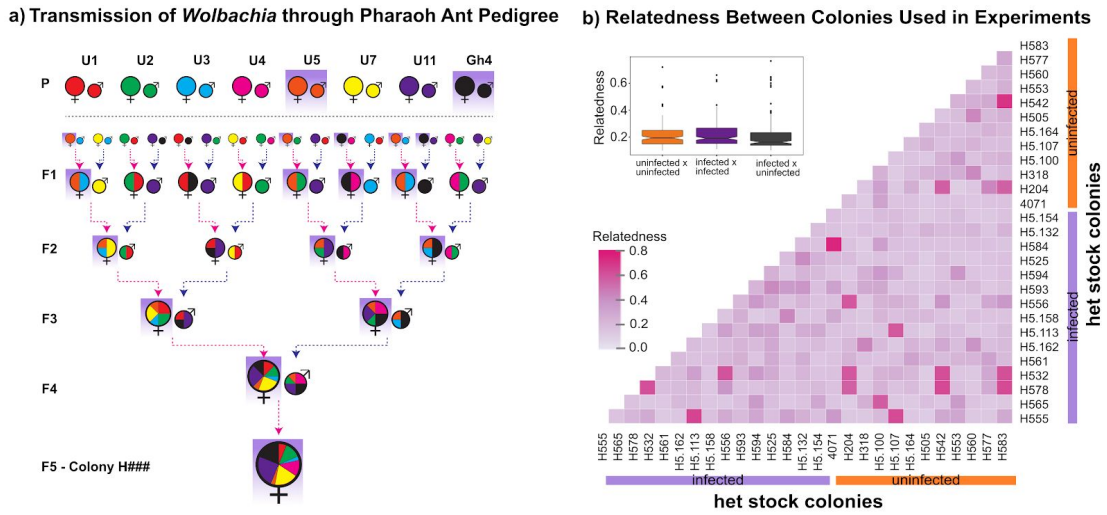

Figure S2.1. *Wolbachia* transmission through pedigree and relatedness amongst pharaoh ant colonies. (a) Schematic representation of intercrossing between eight parental lineages and their subsequent daughter colonies for nine generations to create a single colony 'H###' (H### representing unique colony ID) in the 5th generation. Similarly, crosses were used across nine generations to produce genetically diverse pharaoh ant het stock colonies,some of which have been used as source colonies in the current study (adapted from (J. Walsh et al. 2019)). *Wolbachia* infected queens (females) are highlighted with purple boxes since only queens transmit infection across generations. (b) Genetic relatedness between heterogeneous stock pharaoh ant colonies used to create source colonies in the current study. These heterogeneous stock lab colonies were created following a similar crossing scheme as represented in (a). X and Y-axis of the matrix represent heterogeneous stock colony ID's. The inset box

plot represents the distribution of raw values across three types of plausible heterogeneous stock colony pairs during crossing.

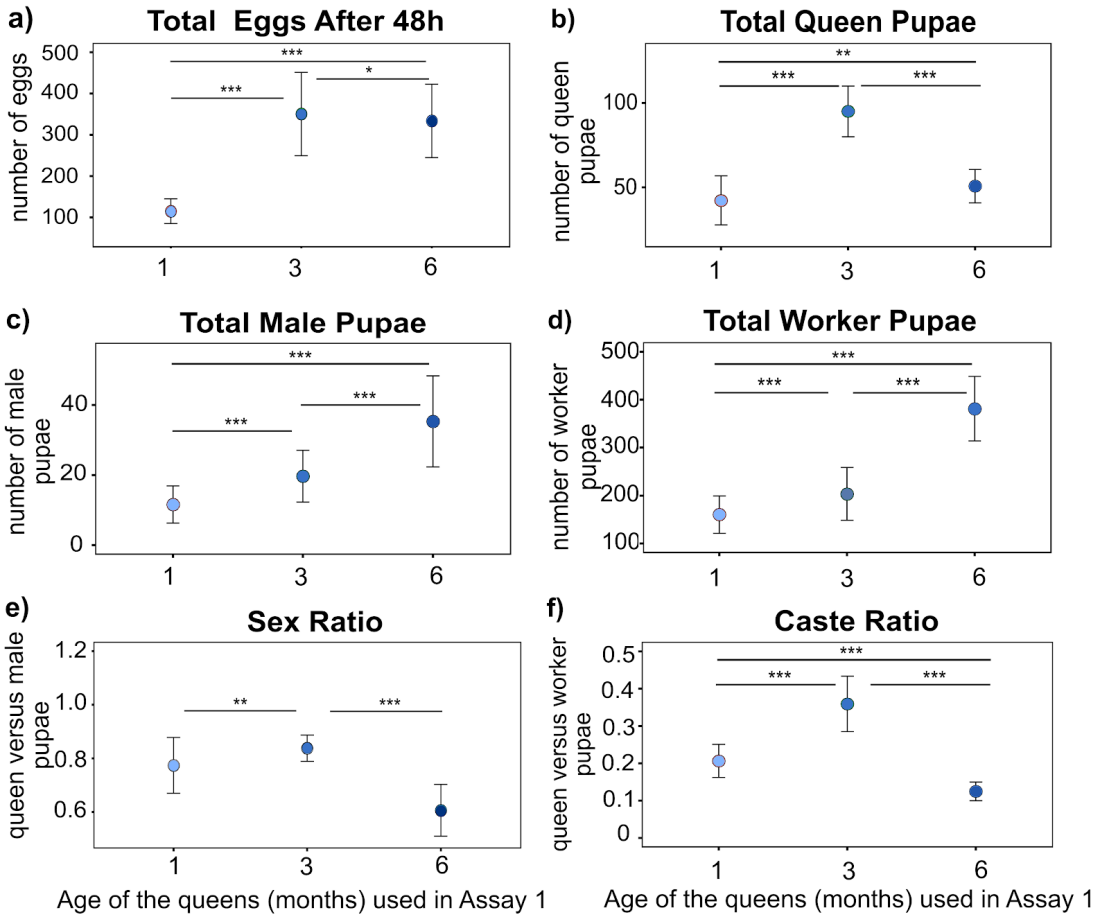

Figure S2.2: Colony-level fitness traits vary across queen age. (a) One month-old queens laid the least number of eggs within 48h. (b) Colonies with three months-old queens produced the highest number of queen pupae. (c) Male production increased as the queens became older. (d) Worker production increased as the queens became older. (e) Male biased sex ratio in older queens. (f) Colonies with three months-old queens had the higher queen-biased caste ratio. X- axis represents the discrete queen ages used in Assay 1, Y-axis represents the trait value, filled circles represent the mean trait value and error bar represents the 95% confidence interval. Statistical differences,

as estimated by TukeyHSD of GLMM for effect of queen ages, are represented by *p < 0.05, **p<0.01 and ***p<0.001. 16 colonies were analyzed per time point.

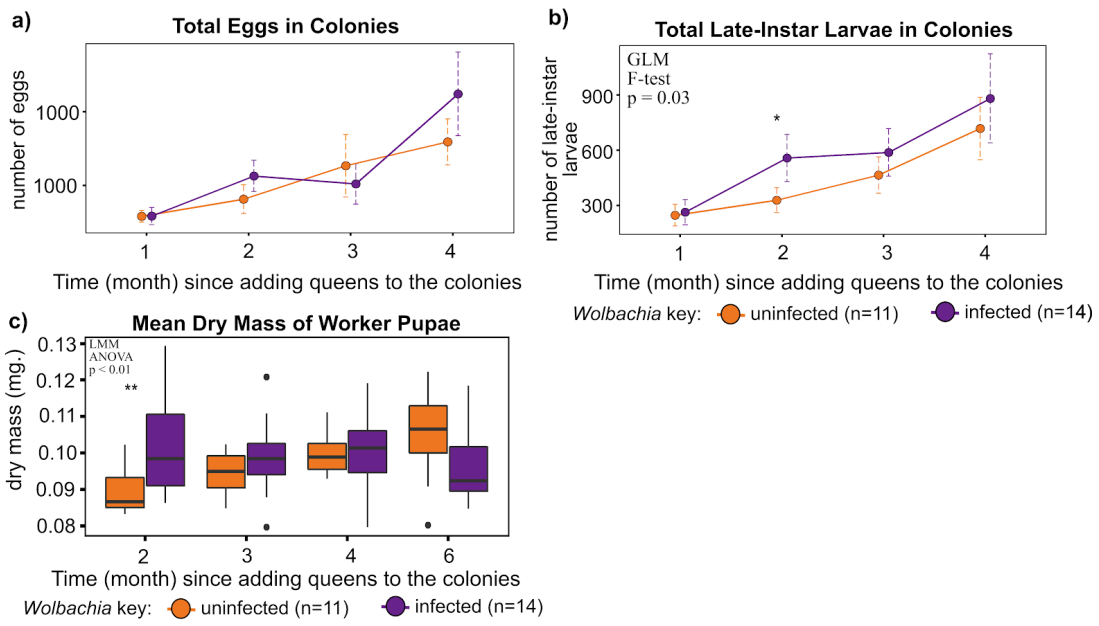

Figure S2.3: Growth dynamics of the early developmental stages in colonies and dry mass of worker pupae. (a) Infected and uninfected colonies produced a similar number of eggs. (b) Infected colonies had a higher number of late-instar larvae after 2 months of adding queens to experimental colonies. (c) Infected worker pupae were heavier after 2 months of starting Assay 2. X-axis represents the time, in months, since Assay 2 was started, Y-axis represents the trait value. For (a) and (b), filled circles represent the mean trait value and error bar represents the 95% confidence interval. *Wolbachia*-driven difference is represented as $*p < 0.05$, and was estimated by age-specific GLM. For (c) Y-axis represents the trait value. *Wolbachia*-driven differences are represented as $**p < 0.01$, which was estimated by ANOVA of age-specific LME. *Wolbachia* color key, along with the number of colonies in the assay (n), are at the bottom of the figure panel.

CHAPTER 3: *Wolbachia*-infected pharaoh ant colonies have higher stage-specific egg production, metabolic rate, and worker survival

Abstract

Wolbachia is a widespread insect endosymbiotic bacteria that has diverse phenotypic effects on its host, ranging from mutualistic to parasitic. *Wolbachia* is also prevalent across ant species, however, its phenotypic effects are not well characterized. We previously found that *Wolbachia*-infected colonies of the invasive ant, *Monomorium pharaonis*, have increased growth and reproductive investment, with possibly increased reproductive senescence of infected queens.

Here we dissect the benefits and costs of *Wolbachia*-ant symbiosis, by comparing the egg-laying rates of queens across their lifespan, the metabolic rates of colonies and colony members at different stages of the colony life cycle, and the survival of queens and workers. Newly-mated infected queens laid more eggs than uninfected queens. Colony-level metabolic rates of infected and uninfected colonies were similar during the early life cycle stages (1- to 2-month-old queens), but infected colonies had higher metabolic rates at later life cycle stages (3-month-old queens) during peak colony productivity. Despite these differences in egg-laying rates and metabolism, infected queens lived as long as the uninfected queens, yet infected workers outlived uninfected workers. Thus, overall *Wolbachia* increased queen egg-laying rate and worker longevity, which act to increase colony-level productivity, without any measurable cost to the host.

Introduction

Wolbachia, a maternally inherited group of alphaproteobacteria, is a widespread insect endosymbiont which is best known for host reproductive manipulation, for example, causing cytoplasmic incompatibility between infected and uninfected mates, killing or feminizing infected males, causing female-biased sex ratio or inducing parthenogenesis (Jan Engelstädter and Hurst 2009; Zug and Hammerstein 2014). *Wolbachia* can also have fitness-enhancing effects, such as increased host fecundity and survival, which are conditional on the *Wolbachia* strain, host genotype, host species, and environment (A. J. Fry, Palmer, and Rand 2004; N. E. Gruntenko et al. 2017; Laurence Mouton et al. 2007; Reynolds, Thomson, and Hoffmann 2003; Zélé, Altıntaş, et al. 2020; N. E. Gruntenko et al. 2019; J. A. White et al. 2011).

Wolbachia also infects an estimated one-third of all ant species (Russell 2012) yet we have limited understanding of the effects of *Wolbachia* on ants and other social insects. The unique biology of eusocial insects, specifically the reproductive division of labor and obligately cooperative lifestyle, may significantly alter *Wolbachia*-induced phenotypes across colony members within a colony and across different colonies. *Wolbachia* infection is correlated with colony dispersal strategy, indicating that *Wolbachia* may affect ant population structure (Treanor and Hughes 2019; Wenseleers et al. 1998; Russell 2012). Infection incidence is higher in colonies with dependent colony foundation, i.e, when new colonies are established by short-distance dispersal of multiple queens and workers from the parent colony, compared to independent colony foundation, when new colonies are established by a queen that disperses over a longer distance. (Treanor

and Hughes 2019; Wenseleers et al. 1998; Russell 2012). *Wolbachia* prevalence varies across different species of ants, across different populations within a species and across different castes within the colonies (Russell 2012; de Bekker et al. 2018; Tsutsui et al. 2003; Reuter, Pedersen, and Keller 2005; Rey et al. 2013; Wenseleers et al. 1998; Shoemaker et al. 2000; Bouwma et al. 2006; Kautz, Rubin, and Moreau 2013; Wenseleers, Sundström, and Billen 2002). *Wolbachia* is lost in the invading populations of the Argentine Ant (*Linepithema humile*; (Reuter, Pedersen, and Keller 2005; Tsutsui et al. 2003)), fire ant (*Solenopsis invicta*; (Bouwma et al. 2006; Shoemaker et al. 2000)), and little fire ant (*Wasmannia auropunctata*; (Rey et al. 2013)). Infected *Formica truncorum* colonies produce less queens and males compared to uninfected colonies (Wenseleers, Sundström, and Billen 2002). Additionally, *Wolbachia* is considered a reproductive manipulator in multiple species of ants (Wenseleers et al. 1998; Shoemaker et al. 2000; Van Borm et al. 2001). Given these reasons, *Wolbachia* was considered a reproductive parasite and detrimental to its ant host. However, as recently shown in the ghost ant, *Tapinoma melanocephalum*, *Wolbachia* can be a nutritional symbiont (Cheng et al. 2019) and thus, may have beneficial effects in some cases.

We previously showed that infected *Monomorium pharaonis* colonies have queen-biased sex ratios (Singh and Linksvayer 2020; L. Pontieri et al. 2017), and higher colony growth and reproductive potential with possibly increased reproductive senescence of infected queens (Singh and Linksvayer 2020). We build on these findings and dissect individual- and colony-level benefits and costs of *Wolbachia* infection in *Monomorium pharaonis*. In the current study, we have assessed *Wolbachia*-driven

differences on the fecundity of queens across their lifespan, the metabolic rates of colonies and colony members at different colony life cycle stages, and the lifespan of queens and workers. With these comparisons we aim to establish if higher colony-level productivity of infected colonies may be explained by higher egg-laying of infected queens and if that has costs that may be specific to certain castes and colony life cycle stages.

Materials and methods

Source of infected and uninfected Monomorium pharaonis *colonies and ant husbandry*

In order to produce a population of colonies with known *Wolbachia* infection status, where genetic background and infection status are relatively uncoupled, we systematically intercrossed colonies that were naturally infected or uninfected with *Wolbachia* for nine generations (Singh and Linksvayer 2020). Next, we separately combined 15 infected colonies and 14 uninfected colonies, to create two sources that differed in *Wolbachia* infection but were genetically similar. We used these sources to create replicate colonies which will be referred to as ‘source colonies’ from hereon (see Singh & Linksvayer 2020 for more information). We experimentally synchronized the age of the queens in these source colonies by removing all existing adult queens from the colonies to initiate production of new queens and males and restart the colony life cycle. This produced queens of known and same age across all the source colonies. These queen age-matched source colonies were used to create experimental colonies used in the current study. All colonies, source and experimental colonies used in the current

study, were reared at 27°C with ± 50% relative humidity, and fed ad libitum synthetic agar diet (sugar:protein = 3:1; (Dussutour and Simpson 2008)) and dried mealworms (*Tenebrio molitor*) twice a week.

Egg laying by newly-mated queens

We first compared the egg-laying rates of newly-mated queens across 20 replicate *Wolbachia*-infected and 11 replicate uninfected groups to establish differences in the early lifespan of the queens which may affect colony growth.

To set up the experimental groups, we collected 50 darkly pigmented queen and male pupae from 20 *Wolbachia*-infected and 11 uninfected source colonies in Petri dishes along with 100 workers from the same source colony ID per Petri dish. We kept the pupae separated by *Wolbachia* infection and biological sex to produce virgin adults. We set up crosses between 20 similarly-aged virgin queens and 15 virgin males of the same source colony ID along with 50 workers in a glass nest chamber inside a fluoned colony box. We labelled these crosses as experimental groups and censused its composition, especially the queens, for five weeks. We used a blind design for the study where we were unaware of the infection status of the experimental colony at the time of census

Egg laying by queens across their lifespan

We also extended a previously published dataset (Singh and Linksvayer 2020) and compared egg laying differences of *Wolbachia*-infected and uninfected queens when the queens were 1-, 3-, 4-, 6- and 9-month-old to establish differences across the

queens' lifespan. Specifically, we created eggless similarly-sized experimental colonies with approximately 500 adult workers, and approximately 500 brood (larvae and pupae). We added 20 queens at the desired queen age from source colonies for 48 hours to these eggless experimental colonies. We then censused the total number of eggs in these experimental colonies to assess the initial differences in egg laying and then returned the queens to their respective source colonies.

Metabolic rate differences between infected and uninfected colonies and colony members

We compared metabolic rates of (a) infected and uninfected whole colonies at two different stages of colony life cycle, and (b) different colony members, namely the brood and the queens, at an early colony life cycle stage to establish *Wolbachia*-, caste-, and colony life cycle-dependent differences.

We estimated metabolic rates using flow-through respirometry (Lighton 2018) on LiCor-7000 for whole colonies and brood, and on LiCor-6252 for groups of queens using the differential gas analyzer mode. We used dry CO_2-free air at a flow rate of 125ml/min (25% of 500 ml/min flow controllers) for whole colonies and brood, and a flow rate of 50 ml/min (100% of 50 ml/min flow controllers) for groups of queens. We used source colonies to create replicate experimental colonies at desired queen age.

We estimated metabolic rates of whole colonies, brood, and queens early in the colony life cycle stage as any differences at this stage may affect growth on a long-term basis. We estimated the metabolic rates of 13 infected and 13 uninfected replicate experimental colonies, each containing 20 1-month-old queens, approximately 250

workers, and 250 brood. We also estimated the metabolic rates of the brood (eggs, larvae, pre-pupae and pupae) from 11 infected and 11 uninfected replicate experimental colonies after recording from the whole colony. We measured CO_2 emission from only one experimental colony per day and alternated between infected and uninfected experimental colonies to ensure that the queens were of similar age between the two groups at the time of measurement. We added a small water tube in the respirometer chamber along with the colony and the brood, to reduce any stress from possible dehydration for the brood.

We also estimated metabolic rates of 14 *Wolbachia*-infected and 15 uninfected groups of approximately 15 queens that were 1- to 2-month-old. We measured one to four groups of queens per day and alternated between infected and uninfected groups of queens to ensure even sampling across queen ages and colony life cycle stages.

We estimated the metabolic rates of eight *Wolbachia*-infected and eight uninfected replicate experimental colonies with 3-month-old queens and approximately 500 adult workers and 500 brood (eggs to pupae). We recorded CO_2 emissions from an infected and an uninfected colony per day. We chose this queen age since *Monomorium pharaonis* colonies peaked in their productivity and *Wolbachia*-infected colonies had increased reproductive investment than uninfected colonies (Singh and Linksvayer 2020). Additional details can be found in the supplementary methods and Fig. S3.1.

Effect of Wolbachia *infection status on the survival of queens*

We compared survival of 18 *Wolbachia*-infected and 16 uninfected groups of 20 queens. We used 2.5-month-old queens from 18 infected and 16 uninfected source

colonies, along with 50 workers to set up experimental groups. We used older queens to compare survival differences since these queens are expected to be invested in and contributing to colony growth. First, we censused the group composition, i.e., eggs, larvae, pupae and adults, once every three weeks and then once a week after four months had passed. We used a blind design for the study, i.e., we did not know the infection status of the experimental group when censusing.

Genetically paired colonies

To further decouple the effect of *Wolbachia* and genotype, we used a reciprocal crossing scheme between six *Wolbachia* infected and six uninfected heterogeneous stock colonies to create pairs of colonies that were genetically similar to each other but one was infected with *Wolbachia* and the other was not (Fig. S3.2). From hereon, we will refer to these colonies as 'genetically paired colonies'. We set up a reciprocal cross using 15 *Wolbachia*-infected virgin queens with 10 uninfected virgin males with 50 workers of the same genotype as the queen and vice versa (Fig. S3.2). We did not cross infected queens with infected males and uninfected queens with uninfected males. These genetically paired colonies were used for comparing worker survival, but were not large enough to set up multiple experimental colonies for other comparisons. More details about these colonies can be found in the supplementary methods section and Fig. S3.2.

Effect of Wolbachia infection status on the survival of workers

We compared the survival probabilities of 23 *Wolbachia*-infected and 25 uninfected groups of approximately 50 (± 5) workers. We used three genetically paired colonies that had both infected and uninfected counterparts and three each of infected

and uninfected colonies without a surviving pair to set up experimental groups. We first collected darkly pigmented worker pupae and 50 workers from three genetically paired colonies per infection group in a small petri dish. Once new workers eclosed from these pupae, we set up at least four replicate experimental groups per unique colony ID. We censused the experimental groups of workers from August 30, 2019 to December 2, 2019 once every three days.

Statistical analysis

We analysed the data in R version 3.6.1 (R Core Team 2019) with car (Fox and Weisberg 2019) and lme4 (Bates et al. 2015) packages for regression analysis and ggplot2 (Wickham et al. 2015) for visualization. We used survival (Therneau and Grambsch 2000) and survminer (Kassambara, Kosinski, and Biecek 2019) packages to compare survival with log-rank tests and Cox proportional hazards, and visualize survival probabilities of experimental groups using Kaplan-Meir method.

We used a generalized linear mixed model framework (GLMM; (Bolker et al. 2009) with poisson error distribution to compare differences in egg laying over time. For this model, we used total number of eggs at each time point as response variable, *Wolbachia* as a predictor variable, number of queens and age of the queens as fixed factors, and experimental colony ID as a random factor to account for repeated measures. We used a generalized linear model framework (GLM; (Bolker et al. 2009) with negative binomial error distribution to assess differences at specific time points with total number of eggs as response variable, *Wolbachia* as a predictor variable, and number of adult workers and queens as fixed factors.

We assessed the allometric relationship between metabolic rates of the whole colonies (microwatts) and mass of the colonies (grams) using a log-log plot (Fig. S3). We estimated metabolic rates, in microwatts and microwatts per gram of the experimental group, from CO_2 levels measured in ppm by assuming an oxyjoule of 19.87 J ml−1 O2 (respiratory quotient of 0.75) and standardized to 25°C assuming a Q_{10} = 2.0 (Lighton 2018). We used a linear model framework (LM) to test the effects of *Wolbachia* infection, queen age, colony-level activity, colony mass, and colony size on estimates of metabolic rates. We computed the test statistic of individual factors in the linear model via ANOVA from the car package (Fox and Weisberg 2019).

Results

Wolbachia-infected pharaoh ant queens lay more eggs early in their life cycle

Newly-mated *Wolbachia*-infected groups of queens produced more eggs over time than the uninfected queens (GLMER: χ^2 = 7.6, p = 0.005; Fig. 3.1a). Specifically, *Wolbachia*-infected groups of queens produced more eggs when the queens were 8-day-old (GLM: χ^2= 4.42, p = 0.035), 23-day-old (GLM: χ^2= 6.82, p = 0.009), 35-day-old (GLM: χ^2= 5.57, p = 0.018), and 50-day-old (GLM: χ^2= 4.81, p = 0.028). However, such egg laying differences were observed only during the early lifespan of the queens (Fig. 3.1b). Colonies with 1-month-old *Wolbachia*-infected queens produced more eggs compared to their uninfected counterparts (GLM: χ^2= 5.88, p = 0.015), while experimental colonies with older queens, did not show significant differences (Fig. 3.1b). It may appear from Fig. 3.1a that egg laying by queens may reduce as the queens approach two months of age, possibly due to death of some queens in the experimental groups. In

reality over time, the egg laying by queens increases (Fig. 3.1b) and is expected to peak around three months of age, as shown previously (Singh and Linksvayer 2020).

Wolbachia-infected colonies have higher metabolic rates depending on the stage of the colony life cycle.

Metabolic rates (microwatts) of whole colonies showed hypometric scaling with mass (Fig. S3.3a) and had a scaling coefficient of 0.58 which is within the expected range (Makarieva et al. 2008; C. R. White and Seymour 2003; Chown et al. 2007). This means that the mass-specific metabolic rate (microwatts per gram) will increase slowly with increasing mass of the ant colony. In contrast to this, the scaling coefficient of metabolic rates (microwatts) of only the brood was 1.1, which suggested that with increase in mass of the brood the mass-specific metabolic rates will increase more than that expected by isometric scaling (Fig. S3.3b). Interestingly for the groups of queens, metabolic rates in microwatts did not show a significant scaling effect with mass of the queens (Fig. S3.3c). Given these relationships between metabolic rates (microwatts) and the mass of the experimental group, we have represented only metabolic rates in microwatts for further discussion.

Wolbachia-infected pharaoh ant colonies with young queens (1- to 2-month-old) had similar metabolic rates as the uninfected colonies (LM: $F = 0.57$, $p > 0.05$; Fig. 3.2a). Metabolic rates of the colonies increased with the mass of the colony (LM: $F = 9.08$, $p = 0.007$) and the mean humidity in the respirometer chamber over the course of CO_2 emission recording (LM: $F = 5.8$, $p = 0.027$). Whereas, *Wolbachia*-infected colonies with older queens (3-month-old) had higher metabolic rates than uninfected colonies (LM: F=

15.6, p = 0.002; Fig. 3.2b) and colony-level metabolic rates increased with colony size (LM: F= 7.98, p = 0.018). We also compared the metabolic rates of different colony members when the colony was in early life cycle stages (1- to 2-month-old queens). Brood (eggs to pupae) from these colonies also did not show differences in metabolic rates when compared to uninfected brood (LM: F= 0.34, p > 0.05; Fig. S3.4a). However, the metabolic rates of the brood increased with the age of the queens that were initially present in the colonies (LM: F= 9.81, p = 0.006), increased with the mass of the brood (LM: F= 7.22, p = 0.016), and showed a marginal increase with the total number of brood (LM: F= 3.22, p = 0.091). Similar to the colonies and its brood, *Wolbachia*-infected groups of 15 queens, that were 1- to 2-month-old, also had similar metabolic rates as the uninfected queens (LM: F= 1.9, p = 0.18; Fig. S3.4b) with no significant interaction of queen age with *Wolbachia* infection (LM: F= 0.98, p > 0.05). The metabolic rates of groups of queens increased with the age of the queens (LM: F= 16.63, p < 0.001), after statistically accounting for variation in mass of the queens.

Caste-specific survival differences due to Wolbachia

Despite differences in egg laying of queens and colony-level metabolic rates at a specific queen age, *Wolbachia*-infected and uninfected queens have similar group- (Log-rank test, p = 0.8; Fig. 3.3a) and individual-level survival rates (GLMM, χ^2 = 0.2, p <0.05; Fig. 3.3b). The estimated median survival of groups was 230 days for *Wolbachia*-infected queens was 230 days and 206 days for uninfected queens (Fig. 3.3a). *Wolbachia*-uninfected groups had a hazard ratio of 1.134 (95%CI: 0.49-2.57).

Within groups, the proportion of alive queens over time was also similar between infected and uninfected groups (GLMM, $\chi^2 = 0.2$, $p < 0.05$; Fig. 3.3b).

Infected workers had a higher group- and individual-level survival than the uninfected workers (Fig. 3.3c,d). Groups of 50 *Wolbachia*-infected workers have a higher estimated survival probability than their uninfected counterparts (Log-rank test, $p = 0.02$; Fig. 3.3c). The estimated median survival of groups was 69 days for infected workers and 57 days for uninfected workers. Groups of uninfected workers had a hazard ratio of 2.04 (95% CI: 1.14-3.75), i.e., uninfected groups of workers were almost twice as likely to die than the infected groups at each time point. Within the group, a higher proportion of infected workers survived over time (GLMM, $\chi^2 = 12$, $p < 0.001$; Fig. 3.3d).

Discussion

We have compared individual- and colony-level life history traits of infected and uninfected *Monomorium pharaonis* colony members and colonies to elucidate the benefits and costs of *Wolbachia* infection. *Wolbachia*-infected queens produce more eggs shortly after eclosing and mating, which does not exact an energetic cost at this early stage of the colony life cycle (1-month-old queens). However, at a later colony life cycle stage (3-month-old queens), when colonies peak in their productivity and reproductive investment (Singh and Linksvayer 2020), colonies have higher metabolic rates. Despite increased egg laying by queens and higher colony-level metabolic costs, *Wolbachia* infection did not trade-off with the queen lifespan. Interestingly, infected workers, which are obligately sterile, outlived the uninfected workers. Thus, increased rate of egg laying by queens and longer lifespan of workers may explain the higher

growth rate and productivity that characterizes infected colonies (Singh and Linksvayer 2020).

With increased egg production by infected queens, we may expect infected colonies to rapidly grow and potentially disperse more. *Monomorium pharaonis* colonies disperse and occupy new nests via dependent colony foundation, i.e., multiple queens and workers “bud” away from a large parent colony and establish a new colony nearby (Fowler, Alves, and Bueno 1993). A higher dispersal rate, if observed in the wild, is expected to be beneficial for *Monomorium pharaonis* especially when invading new habitats. Increased rate of egg production by *Wolbachia*-infected queens may arise because of individual-level differences in the queens, such as increased stem cell differentiation or oogenesis as shown in *Drosophila mauritiana* (Fast et al. 2011) and *Asobara tabida* (Dedeine et al. 2001), and/or differences in the ability of infected workers to rear more eggs. Cross-fostering infected queens with uninfected workers and *vice-versa* will be useful to tease apart the role of queens, workers and queen-worker interaction on *Wolbachia*-induced phenotypes.

Given the increased egg laying by infected queens and increased growth of infected colonies (Singh and Linksvayer 2020), we expected infected colonies to have a higher energetic demand. Furthermore, we also expected this energetic cost to be exacerbated by the maintenance cost of *Wolbachia* (A. J. Fry, Palmer, and Rand 2004; J. A. White et al. 2011; Fleury et al. 2000). However, we did not see differences in metabolic rates of infected and uninfected whole colonies, brood, and queens when the queens were young (1- to 2-month-old). This suggests that *Wolbachia* may offset the

energetic cost of infection and higher productivity, possibly as a nutritional symbiont as shown in the bed bug (*Cimex lectularius*; (Nikoh et al. 2014; Hosokawa et al. 2010)), fruit fly (*Drosophila melanogaster*; (Brownlie et al. 2009)), and ghost ant (*Tapinoma melanocephalum*; (Cheng et al. 2019)). Furthermore, the increased metabolic rates of infected colonies at a later colony life cycle stage (3-month-old queens) may be reflective of the differences in the life history stages of the infected and uninfected colonies. There could at least be two reasons behind this - (a) colony demography, which affects metabolic rates (Shik 2010; Waters et al. 2010; Mason, Kwapich, and Tschinkel 2015), is changing across colony life cycle and/or (b) infected queens are aging faster (see below), possibly as a trade-off with increased egg production at an early age, and this accelerated life history of the infected queens is driving the higher metabolic rates of the colonies at later life cycle stages. Future efforts to compare the metabolic rates of colonies and colony members across multiple colony life cycle stages and environment will be helpful to better understand the energetic costs of *Wolbachia* infection.

We expected infected queens to have a shorter lifespan compared to uninfected queens due to increased investment in egg laying and higher colony metabolic rate at a later colony life cycle stage. However, we did not find any such differences in the longevity of infected and uninfected queens. Since queens are the only egg laying individuals in *Monomorium pharaonis* colonies, the presence of fecund infected queens that live as long as uninfected queens may be beneficial for *Wolbachia* as more infected individuals can be produced over time. On the other hand, infected workers outlived the uninfected workers. *Monomorium pharaonis* shows age polyethism, i.e., age-based task

allocation of workers where younger workers are involved with nursing and older workers are involved with daily high mortality tasks such as foraging (Mikheyev and Linksvayer 2015). Thus, it's possible that in infected colonies, workers are staying longer in each task (e.g., nursing or foraging). As a result, they may be able to provide better care for their colony that can lead to increased colony growth and/or better care for the infected queens that may offset possible costs of infection on queen lifespan. Generally, *Wolbachia* has a variable effect on host longevity depending on *Wolbachia* strain, host species, and environmental conditions (A. J. Fry, Palmer, and Rand 2004). For example, *Wolbachia* infected *Encarsia inaron* (J. A. White et al. 2011) have increased lifespan, infected *Drosophila melanogaster* have reduced lifespan (Min and Benzer 1997), and infected *Drosophila simulans* have similar survivorship as uninfected flies when challenged with a pathogen (Wong et al. 2011).Thus, it's likely that *Monomorium pharaonis* is infected with a fitness-enhancing strain of *Wolbachia* that may underlie the observed phenotypic effects and/or these effects are observable only under the given laboratory conditions.

Investment in reproduction and somatic maintenance, is expected to result in a trade-off between reproduction and longevity (Edward and Chapman 2011; Flatt 2011; van Noordwijk and de Jong 1986), although eusocial insects are an exception to this since only queens reproduce and lay eggs throughout their lifespan while also living much longer than the non-reproducing workers in the colony (Blacher, Huggins, and Bourke 2017; Keller and Jemielity 2006; Parker 2010; Flatt et al. 2013; Keller and Genoud 1997). It is proposed that the cost of reproduction in eusocial insects may be

deferred to the workers that are comparatively shorter lived and perform high mortality tasks, such as defending nests and foraging for food (Korb 2016). *Wolbachia* infection can provide benefits to its host, while exacting a cost (Zug and Hammerstein 2014; Jan Engelstädter and Hurst 2009). However, since *Wolbachia*'s fitness is tied with the fitness of its host, it is expected to evolve reduced costs over time (Weeks et al. 2007). Social regulation of ant colony growth provides a unique opportunity for *Wolbachia* to manipulate individual- and colony-level traits for its own gain. We predict that *Wolbachia* has adapted different manipulation strategies in-line with the role of colony members and their contribution to colony growth, to favor its own vertical transmission. Future experiments assessing the benefits and costs of *Wolbachia* under a variety of environmental conditions, especially stress, will be helpful.

Conclusions

We report that *Wolbachia* is beneficial for *Monomorium pharaonis* colonies, in the tested conditions, as infected young queens produced more eggs, infected colonies had higher metabolic rates at later colony life cycle stages, and infected queens lived as long as the uninfected one, while infected workers outlive the uninfected workers. Such differences in phenotypes, if also observed in the wild, may increase the dispersal rate and invasiveness of *Wolbachia*-infected phenotypes. The differences in patterns of lifespan between infected and uninfected queens and workers suggest that *Wolbachia* may have adapted to exploit the reproductive division of labor, which is a unique feature of eusocial insects, for its own benefit without exacting a tremendous cost on its ant host. Our study also shows that *Monomorium pharaonis* can be a powerful system, including

the possibility to construct genetically paired colonies, to study ant-*Wolbachia* association while controlling for genotypes.

Figures

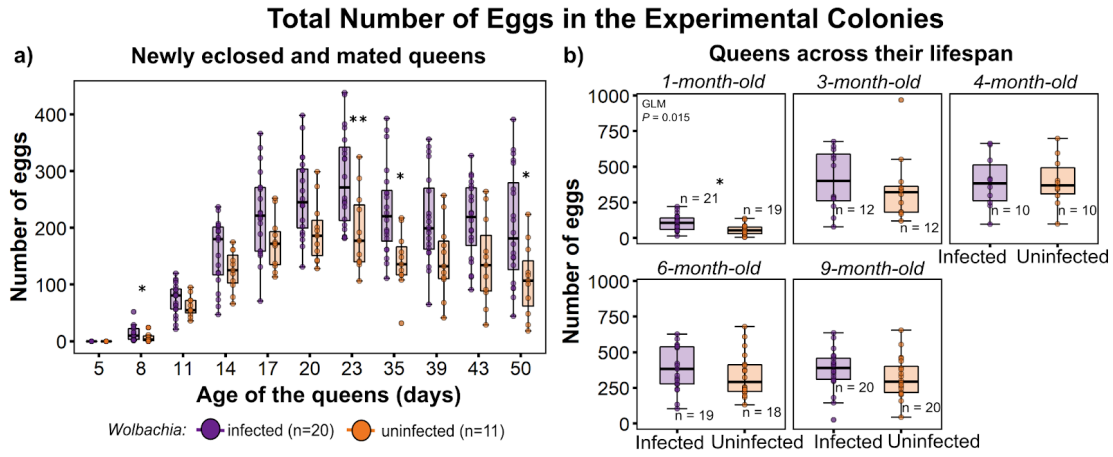

Figure 3.1. *Wolbachia*-infected queens lay more eggs soon after mating. (a) Groups of 20 newly-mated *Wolbachia*-infected queens lay more eggs than uninfected queens. (b) Colonies with 20 1-month-old *Wolbachia*-infected queens laid more eggs after 48 hours of adding the queens to the colonies. However, such differences were not observed when the queens were older. Box plot represents the quartile distribution of the raw data, the filled dots represent the individual raw values. For (a) *Wolbachia* color legend, along with the sample size (n) is included at the bottom of the graph. The x-axis represents the age of the queens in days and the y-axis represents the total counts of eggs in the colonies. For (b) the x-axis represents the *Wolbachia* infection status of the experimental colonies and the y-axis represents the total counts of eggs after 48 hours of adding the queens to the experimental colonies. Sample sizes (n) have been included on individual graphs. Significant differences due to *Wolbachia* infection, as computed from a GLM model, with $P < 0.05$ is represented by '*' and with $P < 0.01$ is represented by '**' on the graphs in (a) and (b).

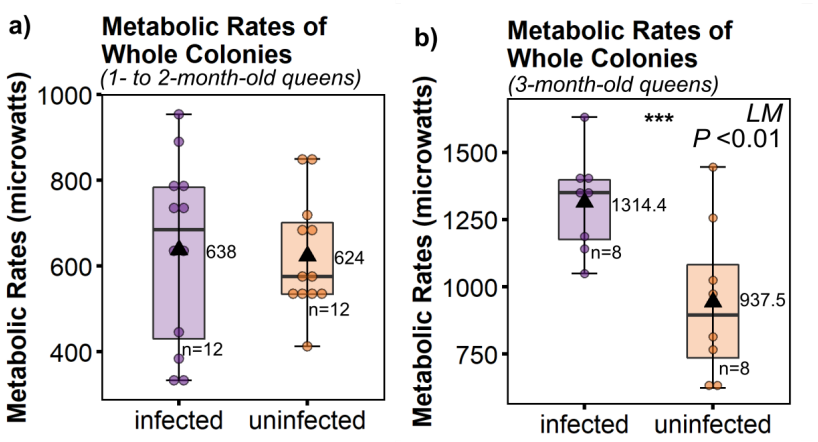

Figure 3.2. Metabolic rates (microwatts) differ between infected and uninfected groups but are dependent on colony life cycle stage and colony component. (a) Similar metabolic rates of whole colonies with 1- to 2-month-old queens which had a source of humidity during CO_2 recording in a respirometer chamber. (d) Higher metabolic rates of infected colonies with 3-month-old queens which did not have any source of humidity in the respirometer chamber. X-axis represents the *Wolbachia* infection status of the experimental group. Y-axis represents the metabolic rates of the groups in microwatts. Box plot represents the quartile distribution of the raw data, the filled dots represent the individual raw values. The filled black triangle in the box plot represents the mean, which is also numerically listed besides the box plot. 'n' represents the sample size for the accompanied box plot. '***' represents the significant difference between infected and uninfected groups, as determined by a linear model, with $P < 0.001$.

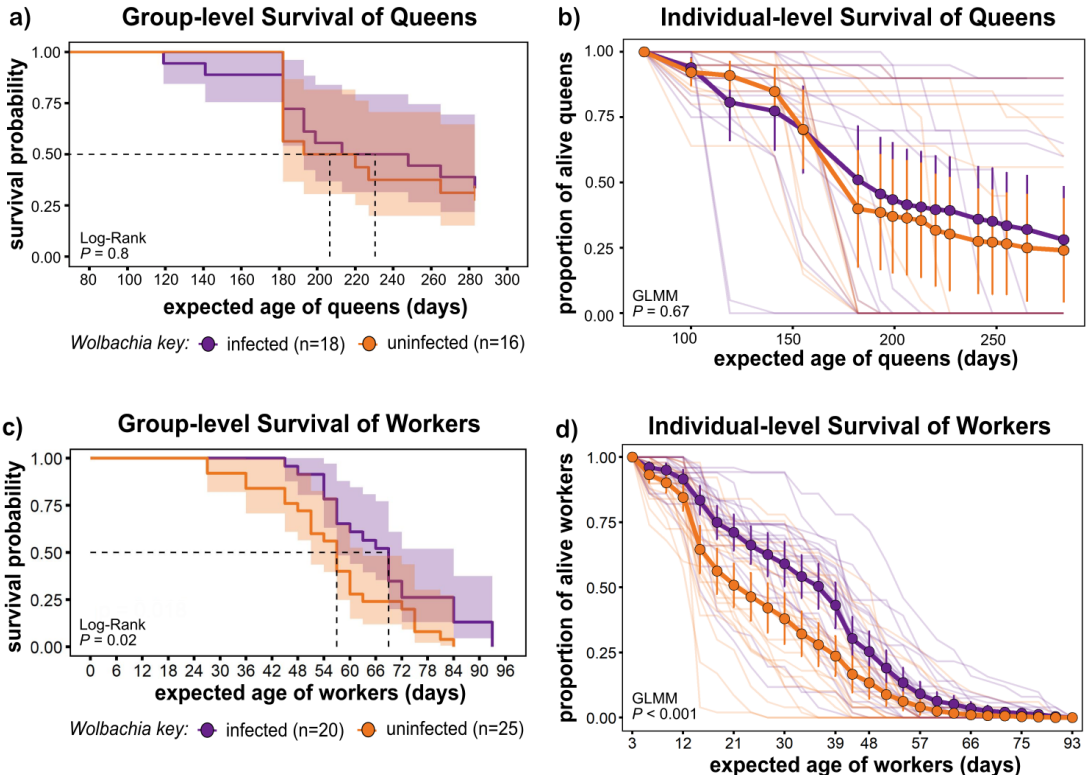

Figure 3.3: Survival differences are dependent on *Wolbachia* infection and caste. (a) Infected and uninfected queen groups have similar survival probability. (b) Infected and uninfected queen groups have similar proportions of alive queens within groups over time. (c) Infected worker groups have a higher survival probability than uninfected worker groups. (d) Infected worker groups have higher proportions of alive workers within groups over time. X-axis represents the estimated age of queens (a, b) or workers (c, d). Y-axis represents the survival probability as estimated by Kaplan-Meier method (a, c) or proportion of alive queens (b) or workers (d). For (a) and (c) solid line represents the mean along with the 95% confidence interval (shaded area). The *P*-value using log-rank test with cox-proportional hazards model is listed on the bottom left corner of the graph. For (b) and (d), filled circles represent the mean value with 95%

confidence interval (error bars). Solid dark line represents the mean trend and lighter lines represent the trend of individual groups. *P*-value estimate from GLMM is listed at the bottom left corner.

Supplementary figures

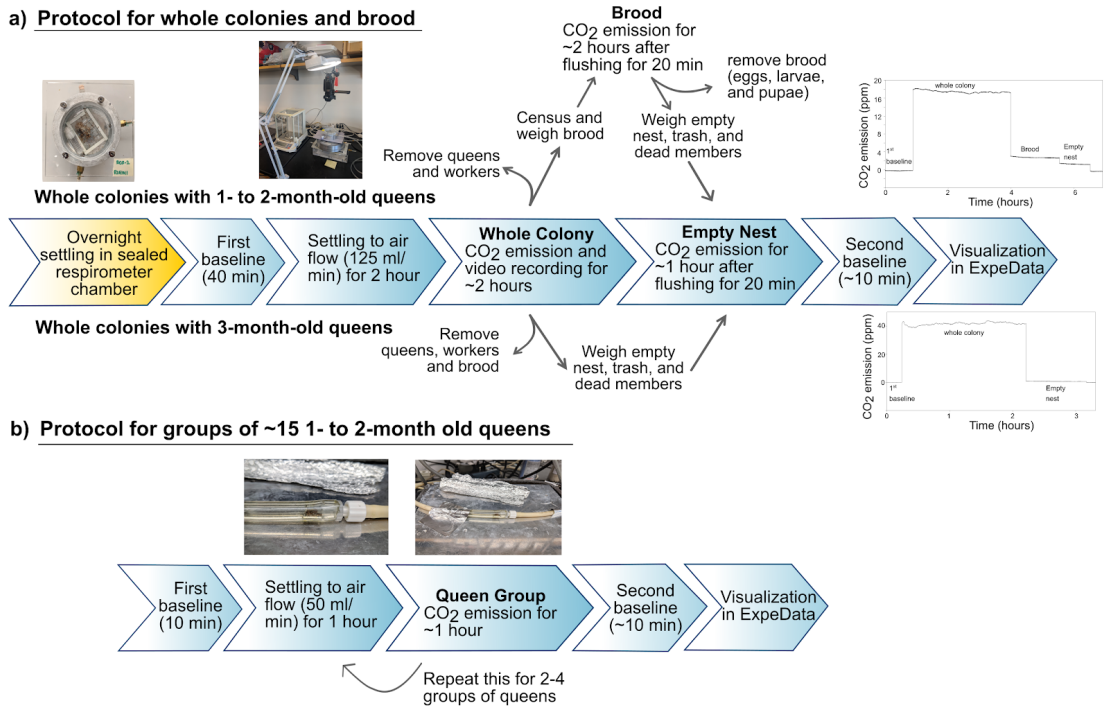

Figure S3.1: Setup used for estimating metabolic rates. (a) Detailed steps for measuring the CO_2 emission from whole colonies and brood with 1- to 2-month-old queens (top half) and whole colonies with 3-month-old queens (bottom half). (b) Detailed steps to measure CO_2 emission from groups of 1- to 2-month-old queens. Yellow color highlights the steps done a day prior to the measurement, whereas the blue color highlights the steps performed on the day of recording CO_2 emission on a respirometer.

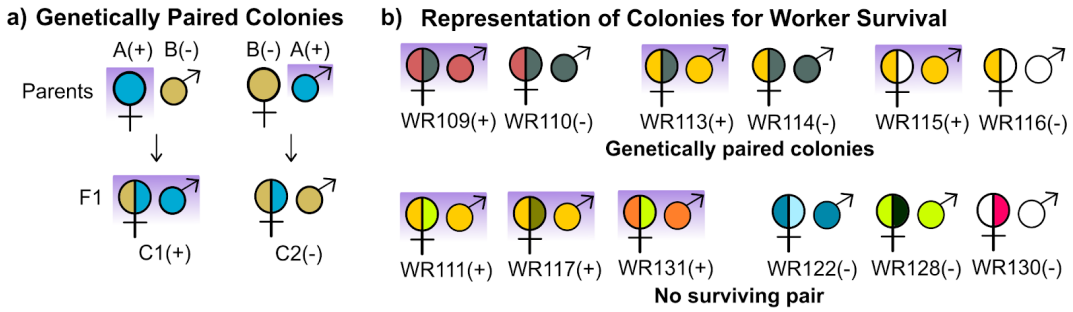

Figure S3.2: Reciprocal crossing scheme to produce genetically paired *Monomorium pharaonis* colonies that differ in *Wolbachia* infection for comparing worker survival. (a) We used a reciprocal crossing scheme to control for genotype when comparing *Wolbachia*-driven differences in life history traits of colonies and colony members. 'A' and 'B' represent sample parent colony ID of differing genotypes and 'C1' and 'C2' represent sample F1 colony ID. (b) A graphical representation of genetic diversity of the colonies used for comparing worker survival. We used 3 pairs of colonies that were expected to be genetically similar but have different *Wolbachia* infection status (top half). We also used colonies that did not have a surviving counterpart (bottom half). Each color represents a unique colony ID from heterogeneous stock colonies used for setting up reciprocal cross. '+' represents that the colony is infected with *Wolbachia*, which is also highlighted by a purple rectangular box. '-' represents that the colony is uninfected.

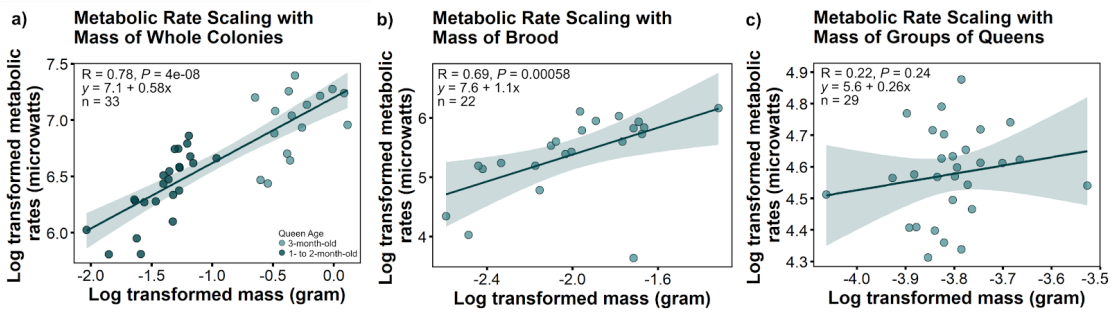

Figure S3.3: Metabolic rate scaling with mass of the experimental group. Log-log plot of metabolic rate with mass of (a) whole colonies with all our data combined (colonies with 1- to 3-month-old queens), (b) only the brood (from colonies with 1- to 2-month-old queens), and (c) groups of approximately 15 queens (1- to 2-month-old). '*R*' represents the Spearman Rank Correlation coefficient, '*P*' represents the significance of correlation, and 'n' represents the sample size. The regression line equation is represented on the top left corner in the format of 'y = x + mc', where 'm' is the scaling coefficient.

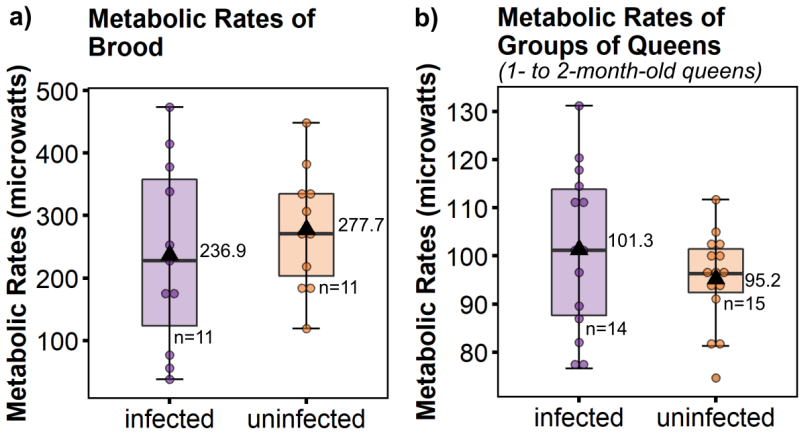

Figure S3.4. Metabolic rates (microwatts) differ between infected and uninfected groups but are dependent on colony life cycle stage and colony component. (a) Similar metabolic rates of brood from the colonies with 1- to 2-month-old queens. (b) Similar metabolic rates of groups of 15 1- to 2-month-old queens. X-axis represents the *Wolbachia* infection status of the experimental group. Y-axis represents the metabolic rates of the groups in microwatts. Box plot represents the quartile distribution of the raw data, the filled dots represent the individual raw values. The filled black triangle in the box plot represents the mean, which is also numerically listed besides the box plot. 'n' represents the sample size for the accompanied box plot.

CHAPTER 4: Fitness-enhancing *Wolbachia* increases in frequency within colonies of the invasive ant, *Monomorium pharaonis*, across generations

Abstract

Wolbachia, a prevalent insect endosymbiont, can spread within its host population, either by manipulating the host reproduction or conferring direct fitness benefits to the host. We previously showed that *Wolbachia*-infected *Monomorium pharaonis* colonies have increased growth rates and reproductive investment than uninfected colonies. In mixed colonies with both infected and uninfected members we may expect *Wolbachia* infection rate to increase over generations but this might be limited by potential trade-offs, such as with host lifespan. We set up three groups of colonies - infected colonies with only *Wolbachia*-infected members, uninfected colonies with only uninfected members, and mixed colonies with both infected and uninfected members in equal numbers - and quantified the within-colony *Wolbachia* prevalence and colony life cycle dynamics over two years, spanning approximately four generations. In the mixed colonies, *Wolbachia* prevalence and queen production increased over time and was higher than the uninfected colonies. The colony life cycle duration was similar across all infection groups, and we found no evidence for fitness costs of infection. Thus, just as fitness-enhancing *Wolbachia* spreads within populations of solitary species, it also rapidly spreads through ant colonies.

Introduction

Wolbachia, a maternally inherited endosymbiotic bacteria, infects over 60% of insect species (Hilgenboecker et al. 2008), although its prevalence within a species can be low (Sazama, Ouellette, and Wesner 2019). *Wolbachia* manipulates it's host's reproductive biology to favor its vertical transmission, such as by inducing unidirectional or bidirectional cytoplasmic incompatibility between infected and uninfected mates, inducing female-biased sex ratio, and killing or feminizing infected males (Jan Engelstädter and Hurst 2009; Zug and Hammerstein 2014). *Wolbachia* can also confer fitness benefits to its host, such as increase in fecundity (Fast et al. 2011; Dedeine et al. 2001), antiviral protection (Hedges et al. 2008; L. Teixeira, Ferreira, and Ashburner 2008), and nutritional provisioning (Hosokawa et al. 2010; Brownlie et al. 2009; Cheng et al. 2019), which may again increase the vertical transmission of *Wolbachia.* These *Wolbachia*-induced phenotypes can incur a physiological cost to its host, such as increased metabolic rates of individual hosts (Evans et al. 2009), reduced host lifespan (Min and Benzer 1997), and reduced survival (Huigens et al. 2004). Depending on the cumulative patterns of *Wolbachia* on host reproduction and survival, *Wolbachia* can spread through host populations, as reported for *Aedes aegypti* (T. L. Schmidt et al. 2017), *Rhagoletis cerasi* (Schuler et al. 2016), and eight sub-groups of *Drosophila* (Michael Turelli et al. 2018).

An estimated 34% of ants are infected with *Wolbachia*, however phenotypic effects of *Wolbachia* on its ant host are largely unclear (Russell 2012; Moreau 2020). Ant colonies pose a unique challenge for *Wolbachia* since colony growth and reproduction

are regulated by different colony members (Cassill et al. 2005; A. M. Schmidt et al. 2011; M. R. Warner, Kovaka, and Linksvayer 2016; Beros et al. 2019). For example, queens lay eggs and contribute directly to colony productivity, whereas workers that are obligately sterile can cull queen- and male-destined eggs and regulate colony demography (Michael R. Warner, Lipponen, and Linksvayer 2018; Børgesen and Jensen 1995; Edwards 1991). It is unknown if and how *Wolbachia* manipulates such within-colony interactions to favor its own transmission and to increase its infection rate within the colony and within the host population.

We previously showed that *Wolbachia*-infected *Monomorium pharaonis* colonies have a queen-biased sex ratio (L. Pontieri et al. 2017) and increased colony growth and reproductive investment (Singh and Linksvayer 2020). Furthermore, as I showed in Chapter 3 of this thesis, *Wolbachia*-infected queens have higher egg laying rates and infected workers have longer lifespan than uninfected workers. Both of these effects are expected to increase colony growth rates. Given these manipulations, we expect *Wolbachia* to spread through the host population. However, we also observed accelerated life cycle and early reproductive senescence (Singh and Linksvayer 2020) of infected colonies which is a potential trade-off. In mixed colonies that have both infected and uninfected members, we expect the later-senescing fecund uninfected queens to prevent the early-senescing infected queens from contributing to the next generation of queens. This is expected to happen since reproductively fecund queens can suppress the production of new queens via pheromonal cues (Oliveira et al. 2020; Van Oystaeyen

et al. 2014). Thus, over time, we expect uninfected queens in such mixed colonies to increasingly contribute to the next generation and *Wolbachia* infection rate to reduce.

In the current study, we experimentally compare the within-colony and within-population infection dynamics in the invasive tramp ant, *Monomorium pharaonis*. We created three distinct groups of colonies, namely, the infected colonies with *Wolbachia* infected members, uninfected colonies with uninfected members, and the mixed colonies with equal numbers of both infected and uninfected members (Fig. S4.1). We document the within-colony dynamics of *Wolbachia* prevalence and dynamics of colony growth, reproduction, and life cycle over two years, corresponding to approximately four generations of *Monomorium pharaonis* colonies.

Materials and methods

We created 12 *Wolbachia*-infected and 10 uninfected Monomorium pharaonis replicate experimental colonies, using previously described source colonies that had similar genetic backgrounds but differed in infection status (Singh and Linksvayer 2020). Each replicate experimental colony had approximately 500 workers, 500 brood (egg, larvae and pupae) and no queens. We also created 12 mixed replicate experimental colonies by adding approximately 250 workers and 250 brood each from infected and uninfected source colonies (Singh and Linksvayer 2020) per experimental colony. All experimental colonies were maintained queenless for 10 days so that eggs in these colonies transitioned to older developmental stages. Once these colonies were eggless, we added 20 1-month-old queens from source colonies (Appendix A1). We added infected queens to infected experimental colonies and uninfected queens to uninfected

experimental colonies. For each mixed colony, we added 10 infected and 10 uninfected 1-month-old queens. Post this we did not manipulate the colonies and recorded observations every 4-5 weeks starting April 2018. All colonies were maintained in environmental growth chambers at 27 ± 1^{o}C, 50% RH and 12:12 LD cycle and were fed ad libitum synthetic agar diet (sugar:protein = 3:1; (Dussutour and Simpson 2008)) and dried mealworms twice a week.

Over time, experimental colonies grew to be sizable and were split in half and moved to a new box after the colony had outgrown three large glass nests (7”L x 3”W). This created a new ‘sub-colony’ for the experimental colony, which was labelled as ‘colonyID-a’, ‘colonyID-b’ and so on, where ‘a’ denoted the original experimental colony and ‘b’ denoted the new sub-colony.

Tracking Wolbachia *dynamics*

We collected at least 12 workers from outside of the glass nest and 12 workers from inside the glass nest, to account for putative differences in colony tasks and/or habitation within the colony due to *Wolbachia*, from each colony in 99% ethanol. We used a quick and easy DNA extraction protocol ((Gloor et al. 1993); Appendix A3) to extract DNA from individual workers and a PCR-based method ((Baldo et al. 2006); Appendix A4) to establish the infection status of up to 24 individual workers per colony as a proxy for colony-level infection prevalence. We sampled after 1,4, 8, 16, 20 and 24 months of starting the assay.

For the first year (1-12 months) we pooled workers from different sub-colonies per experimental colony and stored them as a single sample. We then took 24 workers per colony at random from this pool to determine *Wolbachia* prevalence. For the second year (13-24 months), we separately analyzed workers from sub-colonies to compute the mean infection level per experimental colony.

Tracking colony fitness dynamics

We censused the total numbers of worker pupae, queens, and males, by adding counts from all sub-colonies per experimental colony, to compare colony growth and reproduction dynamics (Fig. S1). We used the counts of worker pupae as a proxy for colony productivity. We used the relative ratio of total number of queens to worker pupae, i.e., caste ratio, as a proxy for colony-level investment in reproduction (queen-biased) or colony maintenance (worker-biased).

We assigned a colony reproductive event as a 15% increase, chosen arbitrarily to account for potential counting errors, in the total number of queens compared to the previous time point. We first computed the mean of the total number of reproductive events across all sub-colonies per experimental colony. We then computed colony life cycle length by dividing the total number of months (24) by the total number of reproductive events per colony.

Statistical analysis

We used R version 3.6.1 (R Core Team 2019) with car (Fox and Weisberg 2019) , lme4 (Bates et al. 2015) , glmmTMB (Brooks et al. 2017), and pscl (Zeileis, Kleiber, and

Jackman 2008) packages for regression analysis, emmeans package (Lenth 2020) for post hoc test, and ggplot2 (Wickham et al. 2015) for visualizations. We used a generalized linear mixed model framework (GLMM; (Bolker et al. 2009) to assess the effect of *Wolbachia* and time on total number of worker pupae, queens and males and caste ratio along with experimental colony ID as a random factor to account for repeated measures. We used a generalized linear model framework (GLM; (Bolker et al. 2009) to assess differences at specific time points. We used TukeyHSD post hoc test for pairwise comparison of the three *Wolbachia* infection groups. We used negative binomial error distribution for count data, binomial error distribution for proportions data that was not overdispersed, and quasibinomial for overdispersed proportions data. For caste ratio, we also included the log of the total number of worker pupae as a fixed factor as colony size regulates caste ratio (A. M. Schmidt et al. 2011). We added '1' to all the counts of worker pupae to have non-zero values for log scale transformation.

We also permuted the proportion of infected workers over time per mixed colony for 10,000 times using the permutes package (Voeten 2019) to statistically confirm the result from the GLMM. For each permutation, we ran a GLMM model to assess the change in infection prevalence.

Results

Wolbachia prevalence increased in mixed colonies (Fig. 4.1a; GLMM: χ^2= 37.66, p < 0.001) with an odds ratio of 1.03 (limits: 1.02-1.04) which implies that with one unit increase in time we expect to see a 3% increase in the odds of increase in infection. This GLMM statistical value was in the top 95 percentile after 10,000 permutations (Fig. S4.2).

Out of the 12 mixed colonies, *Wolbachia* prevalence increased in eight, decreased in two, and was approximately 50% in two of them after two years (Fig. S4.3a). The mean colony life cycle length was similar across infected (approximately 7 months), uninfected (approximated 6 months), and mixed groups (approximately 6 months; Fig. 4.1b; LM: $F = 1.43$, $p > 0.05$).

Over the two years, colonies produced more worker pupae over time (GLMM: $\chi^2 = 345.63$, $p < 0.001$), which differed across the *Wolbachia* groups (Fig. 4.2a; GLMM: $\chi^2 = 8.09$, $p = 0.017$) and the magnitude and direction of growth differences fluctuated across *Wolbachia* and time (GLMM: $\chi^2 = 7.56$, $p = 0.022$). Overall, infected colonies produced more worker pupae than the uninfected colonies (GLMM-TukeyHSD; $Z = 2.98$, $p = 0.008$), whereas mixed colonies showed a variable pattern (Fig. S4.3b). For example, mixed colonies produced less number of worker pupae than infected colonies after six months (GLM-TukeyHSD: $Z = 4.89$, $p < 0.001$) but similar numbers of worker pupae as infected colonies after 20 months (GLMM-TukeyHSD; $Z = 0.78$, $p > 0.05$). At the end of the two years, the number of worker pupae in the mixed colonies did not significantly differ from those in the infected (GLMM-TukeyHSD; $Z = 0.81$, $p > 0.05$) and uninfected colonies (GLMM-TukeyHSD; $Z = 2.02$, $p > 0.05$).

Over two years, colonies produced more queens over time (GLMM: $\chi^2 = 205.23$, $p < 0.001$) that varied across the *Wolbachia* groups (Fig. 4.2b; GLMM: $\chi^2 = 7.31$, $p = 0.025$) and the magnitude and direction of these differences over time depended on *Wolbachia* (GLMM: $\chi^2 = 15.99$, $p < 0.001$). Overall, infected colonies produced more queens than uninfected colonies (GLMM-TukeyHSD; $Z = 2.72$, $p = 0.017$). Queen production trends

for mixed colonies fluctuated over time (Fig. S4.3c), such as, mixed colonies produced less number of queens than infected colonies after four months (GLMM-TukeyHSD; Z = 5.08, $p < 0.001$) but a similar number of queens as infected colonies after 24 months (GLMM-TukeyHSD; Z = 0.07, $p > 0.05$). Despite increased queen production in infected colonies and at times in mixed colonies, we did not observe any differences in the number of males produced by the colonies (Fig. 4.2c, Fig. S4.3d) and the colony caste ratio (Fig. 4.2d; Fig S4.3e) across the three infection groups. Although, after 4 months infected colonies had a higher queen-biased caste ratio than the mixed (GLMM-TukeyHSD; Z = 4.05, $p < 0.001$) and the uninfected colonies (GLMM-TukeyHSD; Z =2.94, $p = 0.009$).

We did not see an overall effect of change in within-colony *Wolbachia* prevalence in the mixed colonies on the total numbers of worker pupae, queens, and males and caste ratio. However, there were time specific effects. For example, mixed colonies with higher *Wolbachia* prevalence produced more worker pupae after 16 months (GLM: Z = 2.38, $p = 0.017$) and produced less queens (GLMM: $\chi^2 = 6.81$, $p = 0.009$) and males (Hurdle: Z-value= -2, $p = 0.045$), and had a worker-biased caste ratio (GLM: F = 6.52, $p = 0.028$) after 20 months.

Discussion

We experimentally studied the dynamics of within-colony *Wolbachia* infection frequency and its consequences on colony-level fitness in the invasive ant, Monomorium pharaonis, for two years by comparing colonies that consisted of approximately 0% (uninfected), 50% (mixed) or 100% (infected) of *Wolbachia*-infected members. *Wolbachia*

spread through the mixed colonies which led to an increase in the production of new queens over the course of two years, corresponding to approximately four generations of *Monomorium pharaonis* colonies. Such fitness benefits are in-line with our previous observational study (Singh and Linksvayer 2020). Additionally, we did not observe reduced reproductive lifespan of the infected queens, since colonies had similar life cycle length irrespective of the colony-level infection status. Thus, under the laboratory conditions, it's possible that fitness-enhancing *Wolbachia* may not exact a cost on its host. If such fitness benefits are also observed in the wild, then we may expect *Wolbachia* to also spread through the ant host populations.

However, *Wolbachia*-induced phenotypes are often conditional on environmental factors which may limit its spread in the natural populations. For example, *Wolbachia* titres and *Wolbachia*-induced phenotypes are temperature sensitive (Hurst et al. 2000; Hague et al. 2020; L. Mouton et al. 2006; S. R. Bordenstein and Bordenstein 2011; Charlesworth et al. 2019). Host population dynamics, such as dispersal and migration, also play an important role in regulating the spread of *Wolbachia* infection (Hancock, Sinkins, and Godfray 2011; Jiggins 2017). In ant colonies, the colony dispersal rate and colony founding methods regulate the population structure, which in turn may affect the *Wolbachia* infection rate. For example, *Wolbachia* is more prevalent in ant colonies that have limited dispersal and dependent colony founding (multiple queens and workers bud away from parent colony and disperse short distance to establish a new nest), compared to colonies where an individual queen disperses long distance to establish new colonies, i.e., independent colony foundation (Treanor and Hughes 2019; Russell

2012). Furthermore, it's possible that previously known physiological costs of *Wolbachia*, such as interference with metabolic pathways (Kremer et al. 2009) and reduced host locomotor activity (Fleury et al. 2000), may become apparent in the wild which may further limit the spread of *Wolbachia.* Future research mapping the prevalence of *Wolbachia* in the wild populations of *Monomorium pharaonis* colonies would be helpful to understand the ecological drivers of *Wolbachia* prevalence. This would also be helpful to understand the association of invasiveness of *Monomorium pharaonis* colonies and colony-level *Wolbachia* infection.

We observed some disparity in infection status of individuals and the colonies that they belong to, i.e., presence of uninfected workers in infected colonies and vice versa, and fluctuations in colony-level infection prevalence over time. Some reasons, in the order of likelihood, that may explain this are false positives or negatives in PCRs, sampling of workers from the colony, imperfect maternal transmission (Carrington et al. 2011; Hague et al. 2020), loss of infection (Van Borm et al. 2001) or contamination with other colonies that have a different *Wolbachia* infection status. We currently do not have the resolution to discern the exact cause. Despite this, we observed consistent phenotypic differences between infected and uninfected colonies, that are in-line with our previous findings (Singh and Linksvayer 2020).

Conclusions

We show that fitness-enhancing *Wolbachia* can increase in frequency within colonies in only a few generations. This increase may also be observed in the wild

colonies, but is expected to depend on a variety of factors such as initial prevalence of *Wolbachia*, fluctuating environment, and physiological costs to the host.

Figures

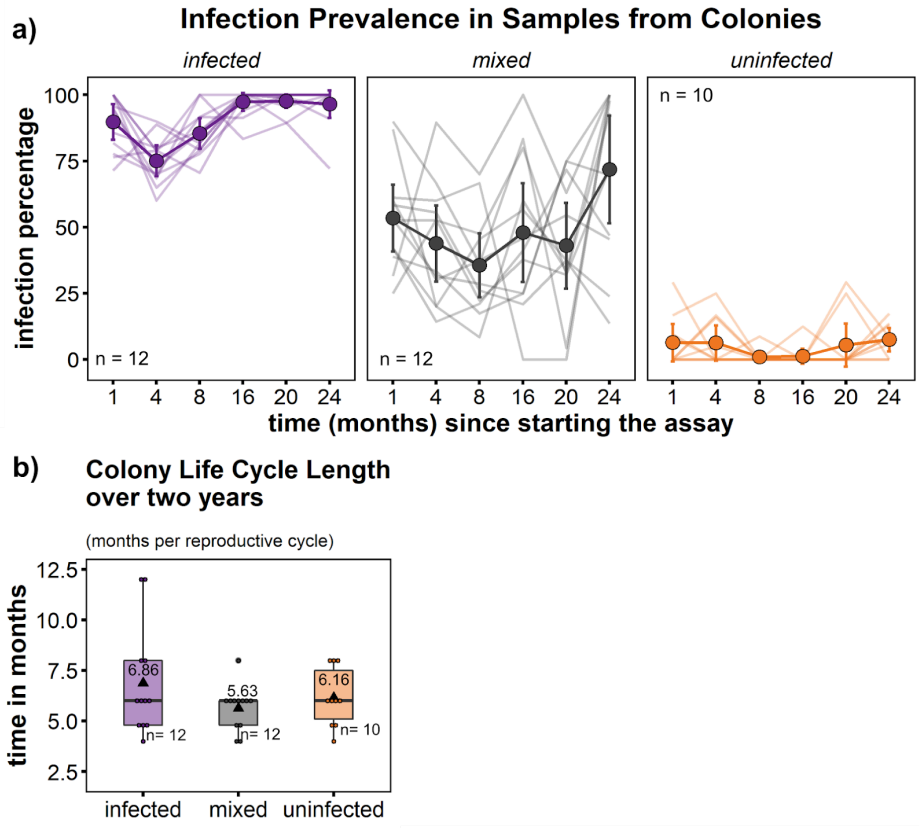

Figure 4.1: *Wolbachia* prevalence increased in mixed colonies and colony life cycle length was similar. (a) Infected (purple) and uninfected (orange) colonies show fidelity to their infection group over time. Infection prevalence in the mixed colonies (gray) increased towards the end of the second year. X-axis represents the time, in months, since starting the assay. The Y-axis represents the infection prevalence as percentage of infected workers in the samples per experimental colony. Light colored lines represent the trends of individual experimental colonies. 'n' represents the sample size per infection group. Filled circles represent the mean value and error bars represent the 95%

confidence interval of the mean.Infection group is listed above each plot in italics. (b) Colony life cycle length did not vary across the three *Wolbachia* infection groups. X-axis represents the *Wolbachia* infection groups of experimental colonies and y-axis represents the life cycle length in months. Filled circles represent the life cycle length of individual colonies. Box plots represent the distribution of these life cycle lengths. Black triangle represents the mean colony life cycle length per infection group. These mean values have also been included either inside the box plot or right outside the box plot. ‘n’ represents the sample size per *Wolbachia* infection group.

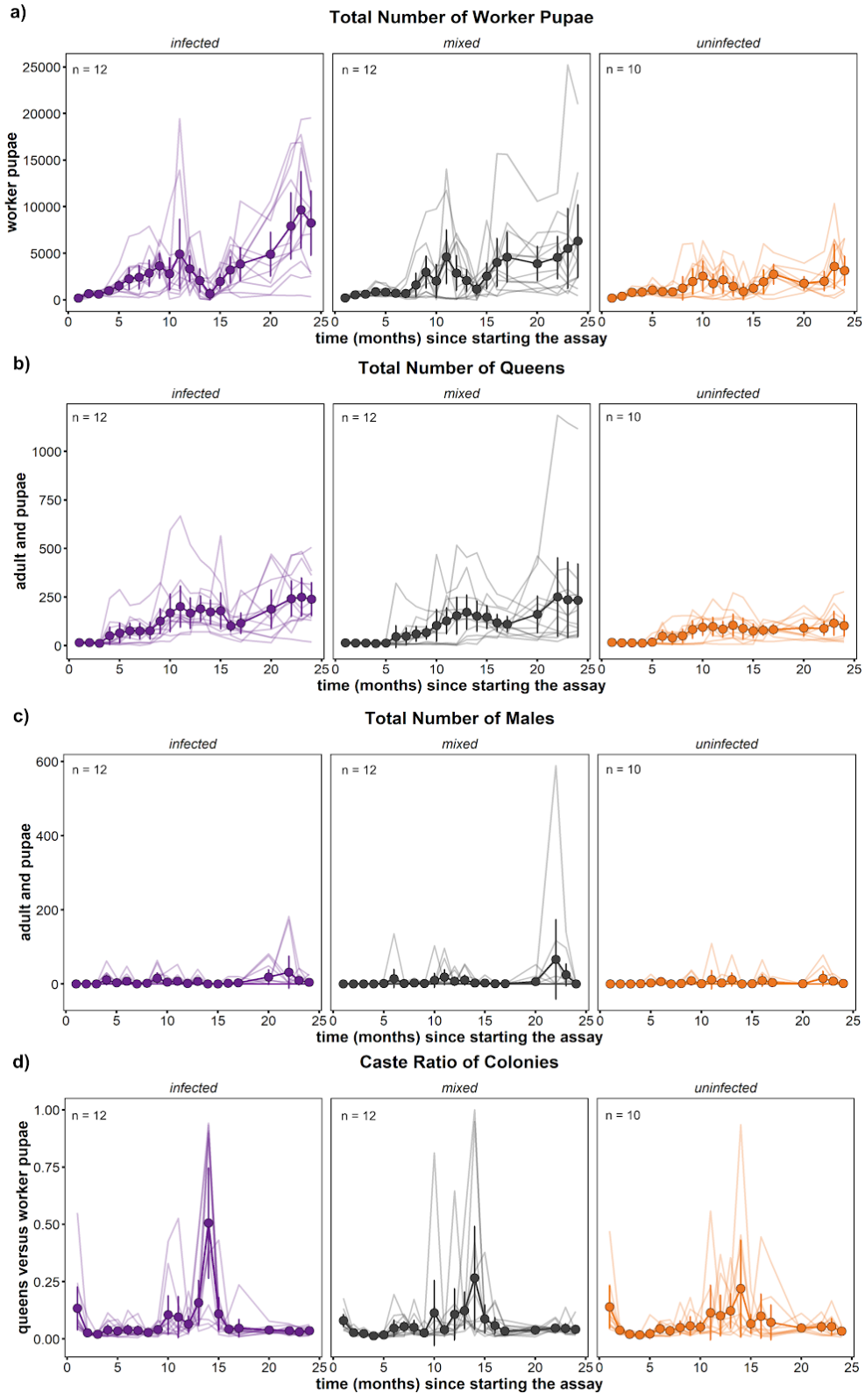
a)
Total Number of Worker Pupae
infected
mixed
uninfected
n = 12
n = 12
n = 10
worker pupae
time (months) since starting the assay
b)
Total Number of Queens
adult and pupae
c)
Total Number of Males
d)
Caste Ratio of Colonies
queens versus worker pupae

Figure 4.2: Colony growth and reproduction dynamics. We have censused different developmental stages to assess colony and reproduction over the course of two years, namely (a) worker pupae, (a) total number of queens as adults and pupae, and (c) total number of males as adults and pupae. Using these census values we were able to compute the caste ratio (d) which represents the relative investment of colonies in reproduction over colony maintenance.X-axis represents the time, in months, since starting the assay. The Y-axis represents the colony fitness measures, such as counts of worker pupae,total number of queens and males, and the caste ratio. Light colored lines represent the trends of individual experimental colonies. ‘n’ represents the sample size per infection group. Filled circles represent the mean value and error bars represent the 95% confidence interval of the mean.Infection group is listed above each plot in italics.

Supplementary figures

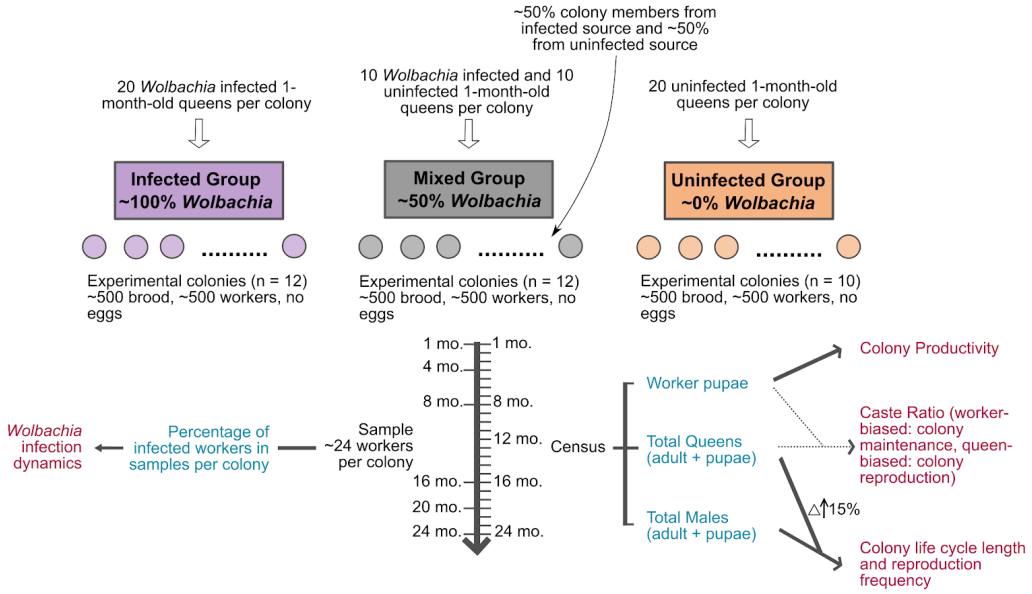

Figure S4.1: Outline of experimental design. We compared the dynamics of colony growth and *Wolbachia* prevalence over two years of three experimental groups - infected, uninfected, and mixed. Infected colonies only had infected individuals (queens, workers, and brood), whereas uninfected colonies only had uninfected individuals. Mixed colonies consisted of both infected and uninfected individuals in equal numbers. Before starting the experiment, we added 20 1-month-old queens to queenless and eggless experimental colonies. Post this we censused these colonies on a monthly basis (represented by ticks on the right side of the arrow) and sampled approximately 24 workers per colony at regular time intervals (represented by ticks on the left side of the arrow). Using these census counts we assessed the colony growth and productivity, reproductive investment, and colony life cycle dynamics. Using sampled workers, we assessed the percentage of infected workers in the sample as a proxy for colony-level

Wolbachia prevalence. The experimental measures are represented in blue text and colony-level traits and inferences are represented drawn from experimental measures are presented in pink text.

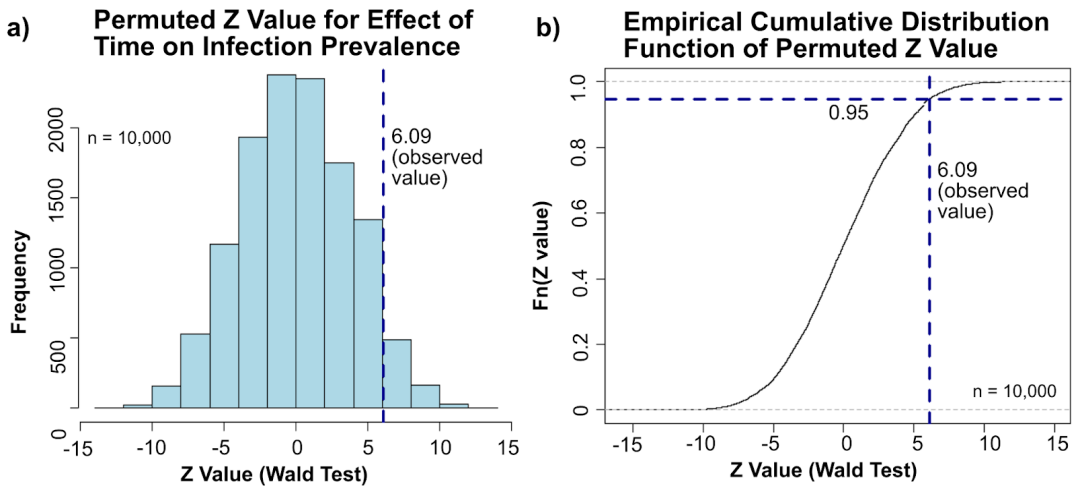

Figure S4.2: Permutation test for change in infection prevalence in mixed colonies over time. (a) Distribution of Z value (Wald Test) of GLMM assessing the increase in *Wolbachia* prevalence over time for each permutation. (b) The distribution of permuted and observed Z value (Wald test). X-axis represents the test statistic of the GLMM to compare the change in *Wolbachia* prevalence over time. The observed value is included on the plot in (a) and (b) and has also been marked with a dashed blue line. For (a), y-axis represents the frequency of occurrence of Z value in the permuted dataset. For (b) y-axis represents the function of the Z value. ‘n’ represents the total number of permutations.

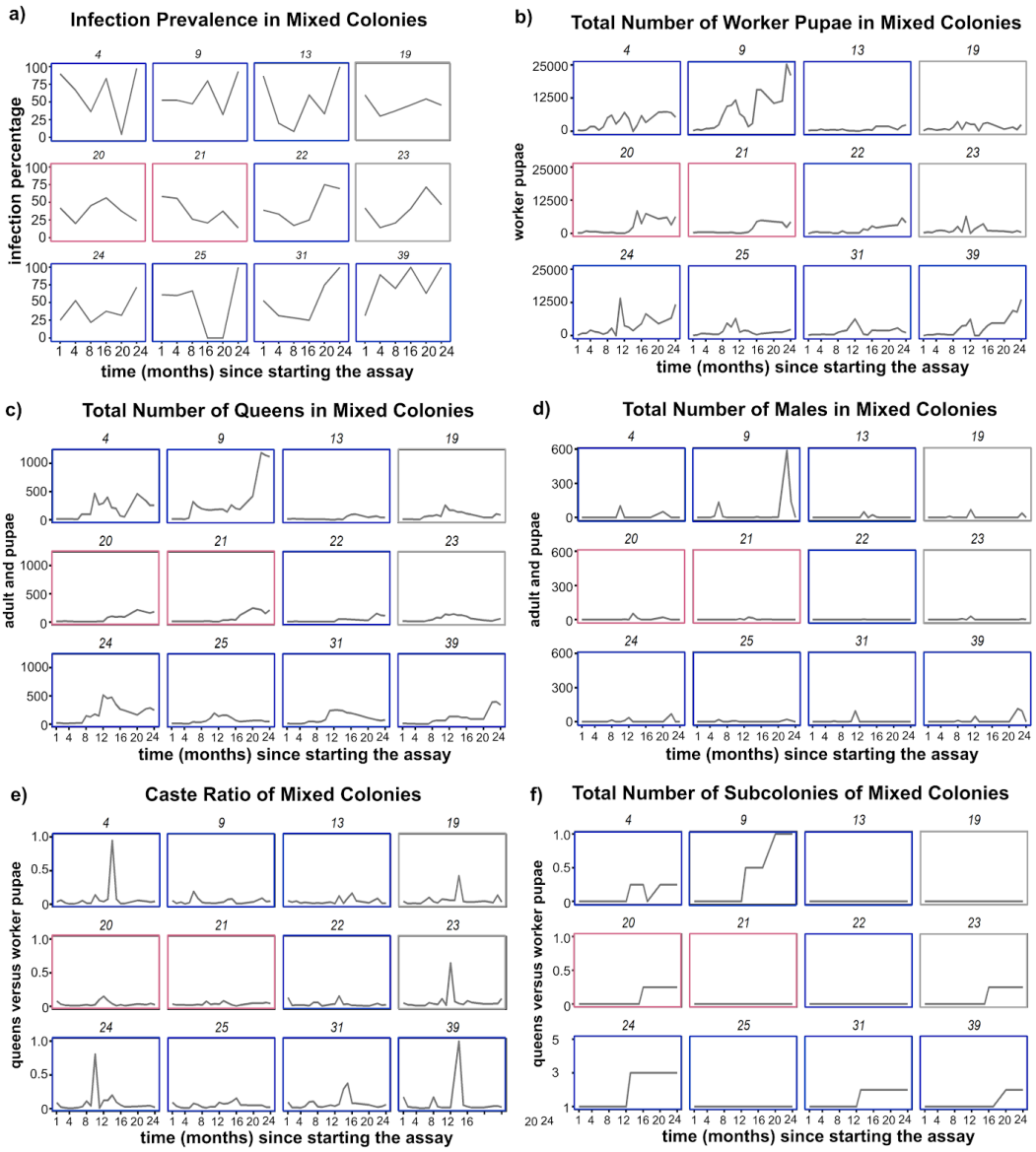

Figure S4.3: Infection and colony growth dynamics of the mixed group. (a) Colonies categorized as 'mixed' at the start of the experiment show fluctuations in infection prevalence over time and variation in prevalence across colonies. (b) Total number of worker pupae. (c) Total number of queens, adults and pupae. (d) Total number of males, adults and pupae. (e) Caste ratio, i.e., the relative number of total number of queens to

total number of worker pupae. (f) Total number of sub colonies over time per experimental colony in the mixed group. X-axis represents the time, in months, since starting the assay. The y-axis represents the trait that is measured. Numbers above the plot in italics represent the colony ID. Blue boxes highlight colonies with >65% infected samples after 24 months, gray boxes highlight colonies with ~50% infected samples after 24 months, and pink boxes highlight colonies with <25% infected samples after 24 months.

CHAPTER 5: CONCLUSIONS

Thesis summary

Eusocial species, such as ants, are an epitome of social living and are characterized by reproductive division of labor, obligately cooperative lifestyle, and overlapping generations within each colony. The colony members extensively interact with each other and these interactions affect the individual-level traits and also drive the colony-level output. For example, colony growth is affected by individual differences in the egg laying rates of the queens and also the ability of non-reproductive workers to cull these eggs. Such social regulation of colony growth of eusocial species presents a unique opportunity for endosymbiotic bacteria, such as *Wolbachia*, to manipulate the individual- and colony-level traits to favor its own vertical transmission. In turn, the host's social interactions may also regulate the endosymbiont-induced phenotypes.

In my thesis, I characterize the individual- and colony-level benefits and costs of a widespread insect endosymbiont, *Wolbachia*, in a highly social ant host, *Monomorium pharaonis*. I also test the evolutionary consequences of these effects, both for the ant host and the endosymbiont. I provide evidence for the fitness-enhancing effects of *Wolbachia* in *Monomorium pharaonis* that does not exact detectable costs. This leads to the rapid spread of *Wolbachia* within colonies in just a few generations of *M. pharaonis* colonies.

Social regulation in ant colonies

Ant colony growth and reproduction is a group-level trait and is regulated by different colony members individually, and the interactions amongst them (Michael R. Warner, Lipponen, and Linksvayer 2018; M. R. Warner, Kovaka, and Linksvayer 2016; Børgesen and Jensen 1995; Børgesen 1989; Oliveira et al. 2020; Aron, Keller, and Passera 2001; Clark et al. 2006; Penick and Liebig 2012; Schmickl and Karsai 2018). For example, in *Monomorium pharaonis* colonies, queens are the only egg laying individuals and are capable of transmitting the maternally-inherited *Wolbachia* (Hölldobler and Wilson 1990). The rate of egg laying is influenced not only by the queens but also by late-instar larvae and adult workers in these colonies. Late-instar larvae process the solid proteins for the colony and can boost queen fecundity (M. R. Warner, Kovaka, and Linksvayer 2016; Børgesen 1989; Børgesen and Jensen 1995). Workers regulate the egg-to-adult survival and colony caste allocation by culling queen-destined eggs (Michael R. Warner, Lipponen, and Linksvayer 2018; Børgesen and Jensen 1995; Edwards 1991). It is interesting to note that *Wolbachia* also infects adult workers given that workers are obligately sterile and can not transmit *Wolbachia*. This endosymbiont seems to have adapted its manipulative strategies according to the reproductive role of the colony member, namely the reproductive queen and the non-reproductive worker. *Wolbachia* also appears to successfully exploit within-colony interactions that regulate colony growth and reproduction to favor its own vertical transmission, without exacting a cost on Monomorium pharaonis adults.

A cross-fostering experimental design is a powerful tool to dissect the roles of queens, workers, and the queen-worker interactions on *Wolbachia*-induced phenotypes. My observations comparing the effects of queen versus colony infection status via a cross-fostering design suggest that the infection status of both the queens and other members influence the colony-level productivity (Fig. 5.1a, b) and reproductive investment (Fig. 5.1c). However, this seems to depend on the age of the queens, which is a proxy for the colony life cycle stage. For example, when the queens are young (1-month-old), the infection status of the queen was a major predictor of egg-laying differences (GLM: LRT = 5.69, $p = 0.017$), whereas we did not observe this effect for queens at other ages. These experiments also highlighted the complexity of dissecting interactions and their influence on *Wolbachia*-induced phenotypes. Moving forward, a full-factorial cross-fostering design will be helpful to dissect the roles of individual colony members on *Wolbachia*-induced phenotypes. Such an approach should take into account the infection status of different colony members (queens, brood and workers), the colony size and the colony demography.

Results from the above discussed cross-fostering experiment also highlight its incongruence with the differences in colony-level fitness between infected and uninfected colonies as observed in Chapter 2. This may partly be explained by the conditional effects of *Wolbachia*. *Wolbachia*-induced phenotypes depend on several factors, such as host species (J. A. White et al. 2011; Min and Benzer 1997; Sasaki, Kubo, and Ishikawa 2002), host genotype (A. J. Fry, Palmer, and Rand 2004; Adam J. Fry and Rand 2002; McGraw et al. 2002), *Wolbachia* strain (A. J. Fry, Palmer, and Rand 2004; Reynolds,

Thomson, and Hoffmann 2003), and the environment (Reynolds, Thomson, and Hoffmann 2003; Hague et al. 2020; Charlesworth et al. 2019; L. Mouton et al. 2006). In ants, even slight variations in the colony composition adds an additional layer of noise since different colony members impact colony growth differently, as discussed in the previous section. Thus, it is imperative to control for environmental conditions and the colony composition to ensure replication of *Wolbachia*-induced phenotypes.

Role of environment and costs of infection

Environment plays an important role in regulating the host's phenotype, *Wolbachia* titres, and *Wolbachia*-induced phenotypes (Sicard et al. 2014). For example, *Wolbachia*-induced metabolic provisioning may sometimes only be evident under stress conditions (Brownlie et al. 2009). *Wolbachia* titres reduce as temperatures either increase or decrease and this can result in imperfect maternal transmission and reduction in *Wolbachia*-induced phenotypes (L. Mouton et al. 2006; Hurst et al. 2000; Murdock et al. 2014; Reynolds, Thomson, and Hoffmann 2003; Wiwatanaratanabutr and Kittayapong 2009; S. R. Bordenstein and Bordenstein 2011). Such conditional effects may explain the absence of cost on *Monomorium pharaonis* due to *Wolbachia*-induced phenotypes, as observed in Chapter 3. However, this does not imply that there are absolutely no costs of infection on the ant host.

In solitary species, such as *Aedes aegypti*, *Drosophila melanogaster*, and *Nasonia*, *Wolbachia* manipulates host reproduction, which is a significant cost to its host since it limits its mating success. Although in *Monomorium pharaonis*, we have not observed reproduction manipulation (unpublished data) as queens and males mate with relatively

equal success irrespective of *Wolbachia* infection. Apart from manipulating reproductive behaviors, *Wolbachia* decreases host activity and body size in the parasitoid *Leptopilina heterotoma* (Fleury et al. 2000), reduces the competitive ability of a parasitoid wasp *Trichogramma kaykai* (Huigens et al. 2004), and reduces the life span in *Drosophila melanogaster* (Min and Benzer 1997; McGraw et al. 2002). Additionally, it is possible that *Wolbachia*-induced costs to the host may become evident under stressful conditions due to competition between *Wolbachia* and its host for resources that are expected to become limited, such as reduced survival of *Wolbachia*-infected *Aedes aegypti* under starvation conditions (Ross, Endersby, and Hoffmann 2016). Future efforts studying the *Wolbachia*-induced phenotypic differences across different environments and stress conditions would be helpful to understand the scope of *Wolbachia*'s effect on host phenotype and fitness.

Interaction of *Wolbachia* with host microbiome

A host is an ecosystem for microbes that interact with each other to regulate the host phenotype and their own phenotypes (McFall-Ngai et al. 2013; Adair and Douglas 2017). *Wolbachia*-induced benefits and costs can be elusive due to its interaction with other microbes within the host and their cumulative effect (V. I. D. Ros and Breeuwer 2009; Goto, Anbutsu, and Fukatsu 2006; Semiatizki et al. 2020; Ye et al. 2017). For example, in the parasitoid wasp, *Encarsia inaron*, which doubly infected *Wolbachia* and *Cardinium* there was no evidence of reproductive manipulation even though *Cardinium* and *Wolbachia* are reproductive manipulators when singly infecting the host.

Wolbachia infection can influence the microbiome composition and diversity of its host, as seen in *Aedes aegypti* (Audsley et al. 2018), *Armadillidium vulgare* (Dittmer and Bouchon 2018), and *Drosophila melanogaster* (Simhadri et al. 2017). Conversely, resident microbial communities can influence the *Wolbachia*-induced phenotypes, such as, impediment of vertical transmission of *Wolbachia* in *Aedes aegypti* by the bacterium *Asaia (Hughes et al. 2014)*. Thus, it may be possible that *Wolbachia* affects the microbial communities of *Monomorium pharaonis* and hence result in colony-level fitness differences.

Monomorium pharaonis microbiome has been partially characterized to identify the presence of pathogenic bacteria (M. M. Teixeira et al. 2009; Alharbi, Alawadhi, and Leather 2019). However, there is no evidence for the effect of *Wolbachia* on ant microbiomes, including *Monomorium pharaonis*. I performed 16S rRNA sequencing of infected and uninfected workers from genetically paired colonies, i.e., pairs of colonies that were genetically similar to each other but differed in *Wolbachia* presence, to compare differences in microbiome composition and characterize the microbiome of *Monomorium pharaonis*. Preliminary results show that *Monomorium pharaonis* are host to a variety of bacterial strains, that show differences in abundance and prevalence between infected and uninfected samples (Fig. 5.2). Future efforts to analyze the current dataset and expand the sample size will be essential for characterizing *Wolbachia*-associated changes in the microbiome of the *Monomorium pharaonis* workers

Future directions

My thesis presents robust evidence for consistent colony-level fitness differences between *Wolbachia*-infected and uninfected colonies, driven partly by individual differences in the queens. Future research can be focused on dissecting the molecular mechanisms of observed phenotypes and behavioral regulation of these phenotypes. One way to achieve this is by characterizing the gene expression differences between infected and uninfected queens across their lifespan to identify signatures of increased fecundity, faster aging and nutritional symbiosis. Since *Wolbachia*-induced phenotypes can be dependent on the environment, it will be useful to characterize the colony-level fitness differences between infected and uninfected colonies and the *Wolbachia* infection rate dynamics across different environments, such as range of ecologically relevant temperatures, conditions of starvation, and pathogen challenge. Shifts in microbial communities in the colonies can also regulate *Wolbachia*-induced phenotypes. Thus, it will be insightful to characterize the interactions of *Wolbachia* with the host's native microbial communities. These results will also be helpful in predicting the spread of *Wolbachia* through natural populations. Additionally, efforts to map the *Wolbachia* infection rates in the wild populations of *Monomorium pharaonis* will help better understand the effects of *Wolbachia* on the reproductive success and invasiveness of infected colonies, and the ecological factors driving *Wolbachia* prevalence.

Conclusions

Endosymbionts are a key aspect of the host's biology and can be considered central to the host's life. Mitochondria and chloroplasts are remnants of a very ancient

endosymbiotic relationship. Lateral gene transfer from an endosymbiont to its host genome are fairly common (Husnik et al. 2013; Kondo et al. 2002; Nikoh et al. 2008; Dunning Hotopp 2011; Dunning Hotopp et al. 2007). Thus, endosymbionts in social species have a potential to regulate and possibly drive key aspects of the colonies, including caste allocation and social interactions. However, endosymbiosis is often overlooked in social insects when it comes to understanding the biology of social insects and their social living.

Despite a wide interest in studying the effects of endosymbionts on colony-level traits, there are several difficulties that heavily limit our ability to do so. There is a considerable difficulty in breeding ants and manipulating ant colonies for behavioral observations, especially those that span multiple generations. Furthermore there are limited standing differences in colony-level infection status of endosymbionts, such as *Wolbachia,* in a study sample. *Monomorium pharaonis* overcomes these shortcomings and should be utilized as a study system to investigate not only the life history effects of endosymbionts, but also to understand the makings of a superorganism.

Figures

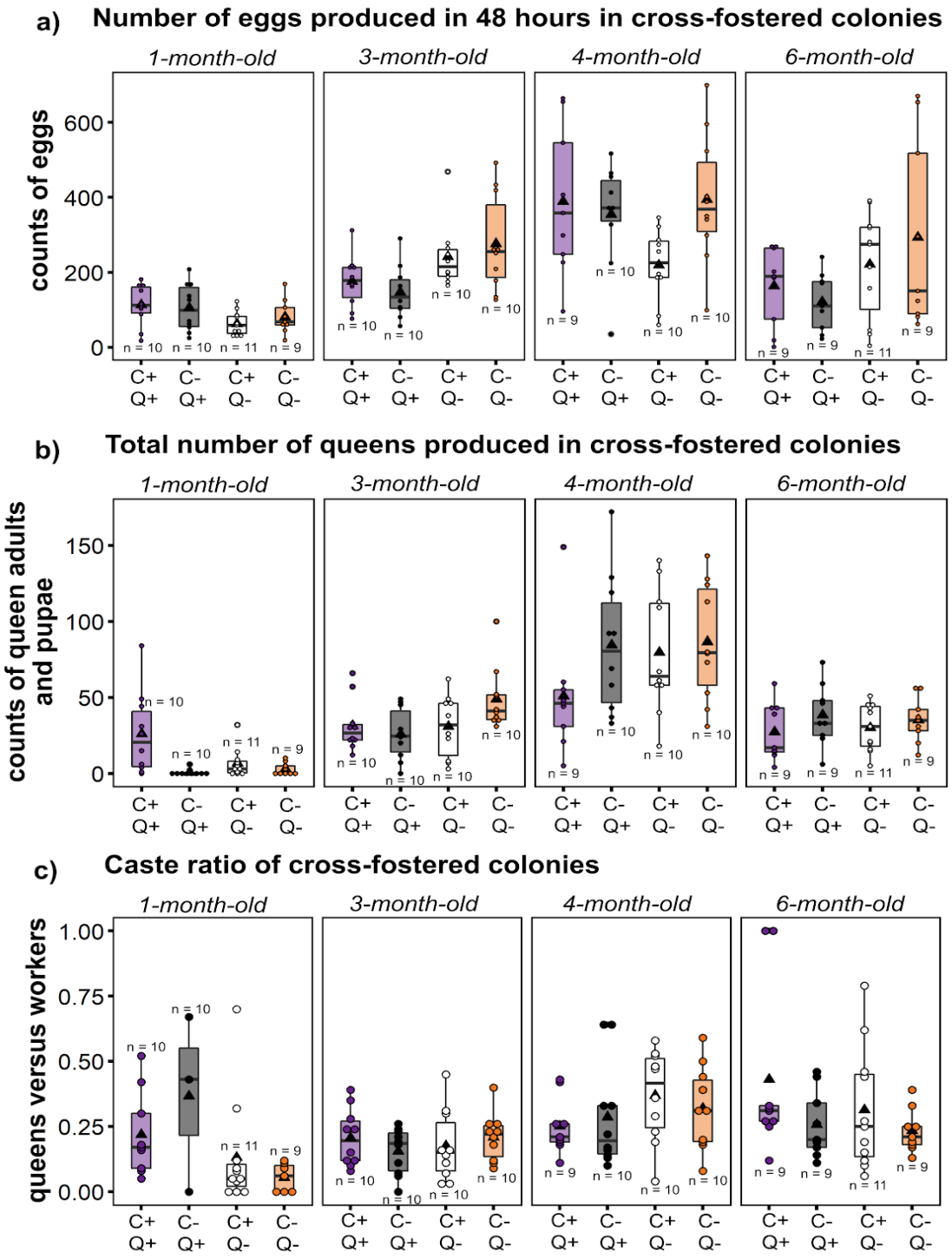
a)
Number of eggs produced in 48 hours in cross-fostered colonies
1-month-old
3-month-old
4-month-old
6-month-old
counts of eggs
0
200
400
600
n = 10
n = 10
n = 11
n = 9
C+ Q+
C- Q+
C+ Q-
C- Q-
b)
Total number of queens produced in cross-fostered colonies
counts of queen adults and pupae
0
50
100
150
c)
Caste ratio of cross-fostered colonies
queens versus workers
0.00
0.25
0.50
0.75
1.00

Figure 5.1: Cross-fostering design to determine the effects of queens and colony members on colony-level fitness. (a) Numbers of eggs in the experimental colonies after 48 hours of adding queens. (b) Total number of queens produced by colonies. (c) Caste ratio, i.e., relative number of queens versus workers produced, of the colonies. The age of the queens used in the experiment is written in italics above each panel. The x-axis represents the cross-fostering design, where 'C' represents colony, 'Q' represents the queens, '+' represents *Wolbachia* infected, and '-' represents uninfected. The y-axis represents the phenotype. 'N' represents the sample size per group.

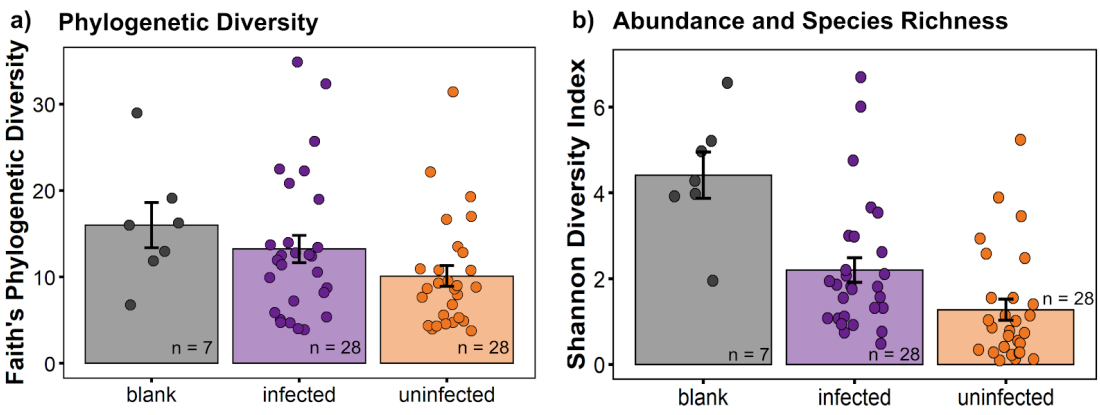

Figure 5.2: Preliminary data representation after 16S microbiome analysis. (a) Phylogenetic diversity of the operational taxonomic units (OTU) represented in the analyzed samples. (b) Species diversity in the OTU in the samples. The x-axis represents the *Wolbachia* status of the samples, where blank had no DNA, infected had DNA from individual infected workers, and uninfected had DNA from individual uninfected workers. The y-axis represents the statistical measure for comparison. 'N' represents the sample size analyzed per group.

APPENDIX

Appendix A1: Source of experimental colonies

We used *Monomorium pharaonis* source colonies, that were genetically similar to each other but differed in their *Wolbachia* infection status, to create experimental colonies for this study (Singh and Linksvayer 2020). Briefly, we separately combined 15 *Wolbachia*-infected and 15 uninfected heterogeneous stock colonies that were genetically diverse to create two separate sources which were then split to create 25 colonies each. These colonies were named ‘source colonies and were genetically similar to each other but differed in *Wolbachia* infection status. These source colonies were also used to produce 1-month-old queens by artificially inducing colony reproduction. We induced colony reproduction by removing all existing queens from the source colonies which allowed the sterile *Monomorium pharaonis* workers to rear new queens and males from the existing pool of eggs. *Wolbachia*-infected and uninfected source colonies and infected and uninfected experimental colonies used in the current study are the same as that used in our previous study (Singh and Linksvayer 2020). More details about the source colonies can be found in Singh and Linksvayer (Singh and Linksvayer 2020) and more details about the heterogeneous stock colonies can be found in Walsh et al. (J. Walsh et al. 2019)

Appendix A2: Analysis of genetic relatedness amongst colonies

We compared the genetic relatedness among the heterogeneous stock lab colonies that were used to create source colonies in the current study. We used genetic

relatedness values from a published dataset from our lab (J. Walsh et al. 2019). We used a permutation test in R using lmPerm (Wheeler and Torchiano 2016) and coin package (Hothorn et al. 2006) to assess if colonies within a *Wolbachia* infection group were more or less related than colonies with different *Wolbachia* infection status. Please refer to Supplementary file S3 for the genetic relatedness values and Dryad for the R script used for this analysis in Singh and Linksvayer 2020 (Singh and Linksvayer 2020).

Appendix A3: DNA extraction from individual workers

We adapted a previously described quick and easy method of DNA extraction from individual fruit flies (Gloor et al. 1993). We made the squishing buffer (SB) as per the protocol (10 mM Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl; (Gloor et al. 1993)) except we added 2000 ug/ml of Proteinase K which was diluted fresh from a frozen stock on the day of DNA extraction. Since workers were collected in 99% ethanol, we first washed them with sterile MQ water and air dried them before transferring individual workers to 1.5 ml microfuge tubes. We then froze the workers in liquid nitrogen and ground them to fine powder in microfuge tubes using sterilized pestle. We then added 50 µL of Squishing Buffer, with Proteinase K, and incubated the samples at 37^{o}C for 30 minutes. We then inactivated Proteinase K by heating the samples to 95^{o}C for 2 minutes. The DNA sample was stored in -20^{o}C till the time of usage for PCR and in -80^{o}C for longer term.

Appendix A4: PCR amplification protocol

We used previously described PCR-based methods for amplifying *Wolbachia*-specific genes in our sample (Baldo et al. 2006). We also used primers for 18S rRNA for pharaoh ants that were designed in the laboratory to test for host DNA as a

validation for DNA extraction (Table S1). For the PCR cycle, we had a 1 min long initial denaturation of DNA at 95^{o} C followed by 35 cycles with 30 sec at 95^{o} C, 30 sec at 53.4^{o}C and 30 sec at 72^{o}C, followed by 10 min extension at 75^{o} C with a final hold 4^{o} C. We used the PCR recipe shown in Table S2. We confirmed the PCR bands on a 1% DNA agarose gel stained with Invitrogen's SYBR safe.

Forward Primer (Sequence)	**Reserve Primer (Sequence)**	**Amplicon Size (Annealing Temperature)**
TL025 (AACAAAGCTTCGCACAATCC)	TL026 (TTTGCTTTTGTGCTGTTTGG)	200 (54ºC)
TL027 (ACAAAGCTTCGCACAATCCT)	TL028 (TTGCTTTTGTGCTGTTTGGA)	198 (54ºC)

Table S1: 18S rRNA primer sequence for Monomorium pharaonis. Primer sequence and annealing temperatures for primer pairs used for amplification of *Monomorium pharaonis* 18S rRNA during PCR.

Reagents	Stock concentration	Reaction concentration	Volume for 1X reaction (µL)
Taq Buffer	10 X	1X	1.0
dNTP mix	10 mM	1.0 mM	1.0
$MgCl_2$	25 mM	2.5 mM	1.0
Forward primer	10 µM	0.8 µM	0.8
Reverse primer	10 µM	0.8 µM	0.8
DNA sample (at least 0.5 µg)			1.5
MilliQ water (sterile)			4.32
Taq DNA polymerase	5 units/ µL	0.04 units	0.08
TOTAL			10 µL

Table S2: Recipe for PCR to test for *Wolbachia* presence. The recipe for PCR reaction for amplifying *Wolbachia* genes and *Monomorium pharaonis* genes from the extracted DNA sample.

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